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Constitutive activation of the Fas/FasL and CD28/CTLA-4 pathways in scurfy mice: implications for peripheral tolerance

Troya Yoder

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To the Graduate Council:

I am submitting herewith a thesis written by Troya Yoder entitled "Constitutive activation of the Fas/FasL and CD28/CTLA-4 pathways in scurfy mice: implications for peripheral tolerance." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Engineering.

Stephen J. Kennel, Major Professor

We have read this thesis and recommend its acceptance:

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To the Graduate Council:

I am submitting herewith a thesis written by Troya Renee Yoder entitled "Constitutive Activation of the Fas/FasL and CD28/CTLA-4/B7 Pathways in *Scurfy* Mice: Implications for Peripheral Tolerance." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

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**Constitutive Activation of the Fas/FasL and CD28/CTLA-4/B7
Pathways in *Scurfy* Mice: Implications for Peripheral Tolerance**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee,
Knoxville

Troya Renee Yoder
August, 1997

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ABSTRACT

Scurfy is an X-linked recessive disorder in mice characterized by the accumulation of activated lymphocytes in the peripheral lymphoid organs, culminating in early lethality due to severe wasting and cytokine toxicity. The gene(s) responsible for the *scurfy* phenotype are unknown, but the massive lymphoproliferation implies a loss of immune tolerance. Although a defect in thymic selection has not been excluded, the phenotype is more consistent with a polyclonal activation of the peripheral immune system. We have examined two pathways that have been documented to maintain peripheral lymphocyte homeostasis, the Fas-FasL pathway and the CD28/CTLA-4/B7 pathway. The Fas pathway is responsible for the activation-induced cell death (AICD) of lymphocytes at the termination of an immune response. It plays a critical role in restoring lymphocyte homeostasis and limiting the potentially deleterious effects of prolonged cytokine secretion. Mice with defects in the Fas-FasL pathway exhibit severe lymphoproliferation and autoimmune disease. In addition to the apoptotic pathway mediated by Fas-FasL, signalling through the CTLA-4 T cell receptor provides a negative regulatory signal to T cells, blocking cell cycle progression. In the absence of CTLA-4, the peripheral immune system is massively activated, leading to a lymphoproliferative phenotype very similar to *scurfy*. Our data suggest that both the Fas-FasL and CD28/CTLA-4/B7 pathways are activated in *scurfy* mice. Although *scurfy* lymphocytes demonstrate evidence of previous and recent activation, have upregulated *Fas* mRNA, Fas cell surface expression, and *FasL* mRNA, we detect negligible levels of apoptosis in freshly isolated

scurfy lymphocytes. It is unclear why activated lymphocytes that are phenotypically-primed for apoptosis are accumulating in *scurfy* peripheral lymphoid organs. However, our data excludes an intrinsic defect in the Fas apoptotic pathway. If provided with an exogenous signal through the Fas receptor, *scurfy* lymphocytes rapidly and spontaneously undergo apoptosis.

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LIST OF ABBREVIATIONS

AICD	activation-induced cell death
APC	antigen presenting cell
Ca ²⁺	calcium ionophore, A23617
CD	cluster of differentiation
cM	centimorgan
ConA	concanavalin A
CRBC	chicken red blood cell
CTLA-4	cytotoxic T-lymphocyte-associated-molecule 4
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
ETn	early transposable element
Fab	fragment antigen binding
Fas	fibroblast-associated
Fas ^{-/-}	Fas knockout mice
FasL	Fas ligand
Fc	fragment crystalline
FCS	fetal calf serum
FSC	forward light scatter
FITC	fluorescein isothiocyanate
G ₀ , G ₁	cell cycle points

<i>gld</i>	generalized lymphoproliferative disorder
GVHD	graft-versus-host-disease
HBSS	Hank's Balanced Salt Solution
IL	interleukin
<i>lpr</i>	lymphoproliferation
<i>lpr^{eg}</i>	<i>lpr</i> complementing <i>gld</i>
mAbs	monoclonal antibodies
MAPK	mitogen-activated protein kinase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
M-MLV	Moloney murine leukemia virus
MRL	mouse strain name
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NOD	non-obese diabetic mice
<i>nu</i>	murine mutation resulting in athymic mice
<i>Otc</i>	ornithine transcarbamylase gene
PE	phycoerythrin
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate

RNA	ribonucleic acid
RNP	ribonuclear proteins
RT	reverse transcriptase
SEB	staphylococcal enterotoxin B
<i>scid</i>	severe-combined-immune-deficient
<i>sf</i>	scurfy
<i>sf spf</i>	scurfy-sparsefur
<i>spf</i>	sparsefur
SSC	side light scatter
TcR	T cell receptor
TNF	tumor necrosis factor
TSS	toxic shock syndrome
WAS	Wiskott-Aldrich Syndrome
WASP	Wiskott-Aldrich Syndrome Protein

Part 1

General Introduction and Background Information

Chapter 1

General Introduction

Scurfy (*sf*) is an X-linked mutation that occurred spontaneously at the Oak Ridge National Laboratory in 1949. The mutation causes a T cell lymphoproliferative disorder that is lethal in hemizygous males by 24 days of age. Although several other mutations cause lymphoproliferation disorders in mice, *scurfy* manifests the most severe disease phenotype described to date. *Scurfy* maps to the centromeric portion of the X chromosome within 2 centimorgans (cM) of *sparse fur* (*spf*), a mutation in the ornithine transcarbamylase gene (*Otc*) (Godfrey *et al.*, 1991; Veres *et al.*, 1987). This region is homologous to human chromosome region Xp21.1-Xp11.23, to which the gene for human Wiskott-Aldrich Syndrome (WAS) maps (Larval and Boyd, 1993). Because of homologous map positions and a number of phenotypic similarities, *scurfy* was a candidate gene for WAS. However, the defective gene, WASP (Wiskott-Aldrich Syndrome protein), was cloned in 1994, and *scurfy* mice show no mutations in the homologous murine gene (Derry *et al.*, 1994; Godfrey, V., personal communication). The causative gene(s) responsible for the *scurfy* phenotype are still unknown.

The *scurfy* disease primarily affects the lymphoreticular system, although there is lymphocyte infiltration of other organ systems. The *scurfy* phenotype is first apparent by a swelling and reddening of the genital papilla at 12-14 days of age, followed by a

scaliness of the ears, feet, tail, and eyelids. The mutants develop graft-versus-host-disease (GVHD)-like lesions, leading to a severe wasting disease that culminates in death by 24 days of age (Godfrey *et al.*, 1991.) The gross pathology of the *scurfy* mouse was described by V.L. Godfrey in 1991 and includes: an enlarged liver with necrotic lesions, scaly skin, anemia, enlarged peripheral lymph nodes, and splenomegaly. A bilobed thymus is initially present, but it is progressively depleted of thymocytes with disease progression and age. Histologically, the *scurfy* mouse is characterized by infiltration of the dermis with a population of pleomorphic leukocytes, complete loss of normal lymph node architecture, and expansion of the spleen by hematopoietic cells. *Scurfy* mice also exhibit a polyclonal increase in serum immunoglobulins, and overexpress mRNA and protein for most cytokines tested (Godfrey, 1991; Blair *et al.*, 1994).

Initial experiments were conducted to examine the role of the thymus in *scurfy* disease. Mice bred to be both *scurfy* and *nude* (*sf/Y; nu/nu*) or *scurfy* and *severe combined immunodeficient* (*sf/Y; scid/scid*) showed no signs of *scurfy* disease. *Nude* mice are born without a thymus, and therefore have no mature T lymphocytes (Stromminger, 1989). *Scid* mice have a defect in DNA repair that results in non-productive rearrangement of T cell receptor and immunoglobulin genes (Stomminger, 1989). Because of this defect, *scid* mice have few mature T or B lymphocytes. The lack of *scurfy* disease in mice with no mature T cells suggested that this population of cells must mediate *scurfy* disease (Godfrey *et al.*, 1991).

The breeding experiments indicated that mature T cells mediate *scurfy* disease, but did not address whether this defect was already present in the precursor stem cell populations of the bone marrow. Therefore, *scurfy* bone marrow was transferred to lethally irradiated congenic or H-2 compatible normal mice. Although donor *scurfy* bone marrow was capable of reconstituting the recipient, *scurfy* disease was not transmitted. From these experiments, it was concluded that *scurfy* is mediated by T cells that must mature in a *scurfy* thymic microenvironment (Godfrey *et al.*, 1991).

Totipotent hematopoietic stem cells migrate from the bone marrow and fetal liver to the thymus where they undergo differentiation into mature T cells. To determine if *scurfy* thymocytes can transfer disease, *scurfy* thymi were grafted to congenic euthymic (immunocompetent) mice, *nude* mice, and *scid* mice. Grafting a *scurfy* thymus to a congenic wild-type recipient did not transmit *scurfy* disease. In contrast, grafting a *scurfy* thymus to congenic *nude* or *scid* recipients resulted in morphological lesions characteristic of *scurfy*. Additionally, injection of *scurfy* thymocytes, lymph node cells, and splenocytes into H-2 compatible *nude* or *scid* mice resulted in transmission of *scurfy* disease (Godfrey *et al.*, 1994). This data was interpreted to mean that *scurfy* disease can be suppressed or inhibited in euthymic recipient mice.

It was established that mature T cells mediate *scurfy* disease (Godfrey *et al.*, 1994). However, this population can be divided into two functionally distinct subsets, distinguished by their expression of the coreceptors CD4 or CD8 (von Boehmer, 1988).

CD4⁺ T cells recognize antigen bound to class II major histocompatibility complex (MHC) molecules. CD4⁺ T cells are considered 'helper' T cells in that their primary function is to secrete cytokines which induce effector functions in B cells and other hematopoietic cells (Coffman *et al.*, 1988). The other T cell subset is defined by the CD8 cell surface receptor. CD8⁺ T cells recognize antigen in the context of class I MHC molecules, and are important in the cytotoxic killing of virally-infected cells and other intracellular pathogens (Teh *et al.*, 1988). To determine if one of these functional subsets was mediating *scurfy* disease, double mutant *scurfy-sparsefur* (*sf spf/Y*) neonates were injected with either anti-CD4 or anti-CD8 monoclonal antibodies (mAbs) to deplete the respective subset population. The double mutants were used because *scurfy* disease is not physically apparent until 12 days, whereas the *sf spf/Y* mutants can be identified by polymerase chain reaction (PCR) assay at birth. Injection of anti-CD8 antibodies, leaving only CD4⁺ T cells, resulted in no change in disease progression. However, depletion of the CD4⁺ subset, leaving only CD8⁺ T cells, ameliorated disease lesions and prolonged lifespan. *Scurfy* disease was not completely prevented in anti-CD4 mAb treated *sf spf/Y* mice because they became immunologically resistant to the mAb treatment. This allowed CD4⁺ T cells to eventually develop and to induce *scurfy* disease. To generate *scurfy-sparsefur* mice completely lacking one T cell subset, the *scurfy-sparsefur* mutation was bred onto β 2-microglobulin (lacking CD8⁺ T cells) and CD4-deficient transgenic mouse lines. β 2-microglobulin-deficient *sf spf/Y* mice exhibited little moderation in disease, where as CD4-deficient *sf spf/Y* mice had prolonged life span and reduced *scurfy* lesions. The final conclusive evidence identifying the CD4⁺ T cell subset as the mediator of

scurfy disease came from adoptive transfer experiments. Adoptive transfer of *scurfy* CD4+CD8- T cells, but not CD4-CD8+ T cells, into H-2 compatible *nude* mice transplanted *scurfy* disease. Therefore, all evidence points to the CD4+ T cell subset as the mediators of *scurfy* disease, although in the absence of CD4+ T cells, other cell types must gain 'helper' functions since even CD4-less *sf* *spf/Y* mice ultimately succumb to *scurfy* disease (Blair *et al.*, 1994). The overall phenotype of *scurfy* mice suggests a massive activation of the peripheral immune system, leading to uncontrolled lymphoproliferation and secretion of toxic levels of inflammatory cytokines, culminating in early lethality.

Research Goals

The goal of this research project was to investigate two pathways that have been identified to play a role in maintaining peripheral lymphocyte homeostasis, the Fas-FasL pathway (described in Part 2) and the CD28/CTLA-4/B7 pathway (described in Part 3). Mice with defects in these pathways exhibit phenotypic similarities to *scurfy* mice, particularly mice in which the CTLA-4 pathway has been completely knocked out (Chu *et al.*, 1993; Takahashi *et al.*, 1994; Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). Therefore we wanted to examine the expression and function of these pathways in the *scurfy* mutant mouse in an attempt to localize the *scurfy* protein within known lymphoid signalling pathways. Relating *scurfy* to defective signalling in the Fas/FasL or CD28/CTLA-4/B7 pathways could help to identify the *scurfy* gene.

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Part 2

Apoptosis in *Scurfy* Lymphocytes

Chapter 1

Introduction

Apoptosis

In multicellular organisms, cell death can be either random or programmed. The morphology of cells undergoing random death is described as necrotic, whereas that of cells undergoing programmed cell death (PCD) is usually, but not always, apoptotic. The term 'apoptosis' is often incorrectly used interchangeably with programmed cell death. PCD originally implied that cell death occurred within a developmental context, and unlike necrosis, PCD required *de novo* gene expression (Schwartz and Osborne, 1993). However, the term apoptosis describes a form of cell death that can be *morphologically* distinguished from necrotic cell death, and may or may not occur in a developmental context. Necrosis describes the morphology of cells dying from massive cellular injury (Lennon *et al.*, 1991). Necrotic cells are characterized by an early swelling of the mitochondria, rupture of the plasma membrane, and subsequent release of the cytoplasmic contents. The release of cellular components into the intercellular space often leads to the inflammatory responses observed after necrosis (Darzynkiewicz *et al.*, 1994). In contrast, apoptotic cells are characterized by nuclear chromatin condensation, no loss of plasma membrane integrity, and activation of an endogenous endonuclease which cleaves internucleosomal DNA linker regions. When examined by gel electrophoresis, the cleaved DNA produces the characteristic DNA "ladder" that has been considered the hallmark of apoptosis (Wyllie *et al.*, 1984). During apoptosis, the plasma membrane pinches off into

membrane-bound apoptotic bodies which are phagocytized before their cellular contents are released. Therefore, apoptosis does not generally lead to inflammation and secondary tissue damage. Because apoptotic cells retain their plasma membrane integrity, they exclude vital dyes that are often used to characterize dying cells (Darzynkiewicz *et al.*, 1992).

The Role of Apoptosis in the Immune System

Central Tolerance

Apoptotic cell death plays a pivotal role in maintaining tolerance and cellular homeostasis in the immune system. This is particularly well documented with thymic-derived or T lymphocytes. It has long been established that central (or intrathymic) tolerance is maintained by the apoptosis of developing thymocytes through the processes of positive and negative selection. Immature T cells that recognize self major histocompatibility complex (MHC) are positively selected, while those that do not, die by apoptosis (Von Boehmer, 1994). Among immature T cells that survive positive selection, those that are strongly reactive to self-components in the thymus are deleted by apoptosis through a process termed negative selection (Nossal, 1994). Immature T cells that survive both positive and negative selection continue to differentiate into mature single-positive CD4 or CD8 T cells which are exported to the periphery. This strict selection process is necessary to release only mature, self-restricted, self-tolerant, antigenically-reactive T cells into the periphery. Evidence suggests that only 5% of developing thymocytes are released into the periphery, while the remainder perish in the thymus (Surh and Sprent, 1994).

Peripheral Tolerance

Once mature T cells migrate to the peripheral lymphoid organs , they are fully capable of responding to antigenic stimulation. In the presence of a costimulatory signal, ligation of the T cell receptor (TcR) of mature T cells, either by foreign antigens or self-antigens that were not encountered in the thymus, results in their activation. The activated T cells proliferate into a large pool of effector clones capable of producing potentially toxic levels of inflammatory cytokines. Therefore, mechanisms must also exist in the periphery to control lymphocyte expansion during an immune response, as well as maintain tolerance to self-antigens encountered in the periphery. This is referred to as peripheral (or extrathymic) tolerance and evidence strongly suggests that this process may proceed through an apoptotic mechanism (Jones *et al.*, 1990).

Until recently, *in vitro* evidence suggested that apoptosis was restricted to the immature T cell populations undergoing differentiation in the thymus, and that mature single-positive T cells were largely resistant to the induction of apoptosis. This view was supported by the fact that many of the same stimuli that induced apoptosis in immature T cells, induced activation and proliferation in mature T cells (Kabelitz *et al.*, 1993). For example, ligation of the TcR of immature T cells with either antigen or anti-CD3 antibodies causes the cells to undergo apoptosis. Ligation of the TcR, of mature peripheral T cells results in their activation and subsequent clonal expansion into effector cells capable of secreting cytokines and stimulating antibody production (Russell *et al.*, 1991). However, it has been discovered that the activation state of a mature T cell plays

a major role in determining its susceptibility to apoptosis. It appears that mature resting T cells are largely resistant to apoptosis, while mature activated T cells are susceptible (Russell *et al.*, 1991). Apoptosis of mature T cells by repeated activation is termed activation-induced cell death (AICD) and is thought to limit the expansion of lymphocytes during an immune response, and to delete autoreactive cells that have escaped thymic selection (Lynch *et al.*, 1995). AICD occurs through an apoptotic pathway mediated by the Fas/FasL association (Van Parijs *et al.*, 1996).

Fas/FasL (*lpr/lpr* and *gld/gld*)

Historically, thymic deletion has been considered to be the primary mechanism involved in the maintenance of immune tolerance. But a number of genes have been identified which are thought to play a role in peripheral deletion, but not thymic selection (Singer and Abbas, 1994). Mice with mutations in these genes have severe immune dysfunction, thereby supporting the crucial role of peripheral deletion in the maintenance of immune tolerance.

Two autosomal recessive, non-allelic mouse mutations, *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease), have been identified that represent defects in a receptor-ligand pair that appear to play a significant role in lymphocyte homeostasis and the activation-induced cell death of mature peripheral T cells (Russell *et al.*, 1993; Singer and Abbas, 1994). The mouse *lpr* locus is found on chromosome 19, and the *gld* locus has been mapped to mouse chromosome 1 (Watanabe-Fukunaga *et al.*, 1992; Takahashi

et al., 1994). MRL-*lpr/lpr* and MRL-*gld/gld* mutants are characterized by autoimmune disease and progressive lymphadenopathy and splenomegaly that are reminiscent of human systemic lupus erythematosus (SLE) (Andrews *et al.*, 1978). The severity of both the autoimmunity and the lymphoproliferation are highly influenced by genetic background, with the most severe phenotype manifested in the MRL-strain (Izui *et al.*, 1984). The modifying genes have not been identified and their role in accelerating autoimmunity is not understood (Cohen and Eisenberg, 1991). In addition, *lpr* and *gld* mice accumulate a non-neoplastic, non-proliferating subset of thymus-derived T cells, referred to 'double-negative T cells'. They are α/β -TcR⁺, but lack both CD4 and CD8 coreceptors, and express the B cell marker, B220. This abnormal subset is detected in the lymph nodes of mutant mice by 4 weeks of age, and may comprise up to 90% of the T cells in the lymph nodes by 6 months of age (Wofsy *et al.*, 1984).

In 1993, it was discovered that the defect in *lpr* mice was a loss-of-function mutation resulting from the insertion of an early transposable element (ETn) into intron 2 of the *Fas* (fibroblast-associated) gene, a gene involved in apoptosis (Chu *et al.* 1993). The ETn contains a polyadenylation signal which causes premature termination of transcription. As a result, very little full-length *Fas* mRNA and surface protein are detectable in *lpr* homozygous mice (Chu *et al.*, 1993). The *gld* mutation is a single amino acid substitution in the Fas ligand (*FasL*) gene, which prevents its binding to the Fas receptor and transducing the apoptotic signal (Takahashi *et al.*, 1994). An additional mutation allelic to *lpr*, *lpr*^{tg}, results from a point mutation in the intracellular domain of the Fas receptor.

This mutation blocks transmission of the apoptotic signal by Fas, resulting in a phenotype very similar to *lpr* and *gld* (Krammer *et al.*, 1994).

Fas is a type I transmembrane protein that belongs to the tumor necrosis factor (TNF) / nerve growth factor (NGF) superfamily of receptors (Watanabe-Fukunaga *et al.*, 1992). Other members include CD27, CD30, CD40, OX40, and 4-1BB, all characterized by cysteine-rich sequences within their extracellular domains that are necessary for ligand binding. The Fas and TNF receptor 1 share homology in a 70 amino acid stretch known as the 'death domain' which is responsible for transducing the death signal (Tartaglia *et al.*, 1993). Fas is expressed primarily on lymphoid cells, as well as on a variety of cell types of hemopoietic and epithelial lineages. In addition, Fas expression is upregulated upon activation (Nishimura *et al.*, 1995). The FasL is a type II membrane protein and is a member of the TNF family, which includes TNF-alpha, LT-alpha, LT-beta, CD27L, CD30L, and CD40L (Suda *et al.*, 1993). The expression of FasL is restricted to activated mature T cells, and is constitutively expressed in the immunologically privileged tissues of the eye and testis (Suda *et al.*, 1995; Suda *et al.*, 1993). Both Fas and FasL can also exist as soluble proteins by proteolytic cleavage of their transmembrane domains, and some forms of soluble Fas are thought to block Fas-mediated apoptosis (Hughes and Crispe, 1995; Cascino *et al.*, 1995; Tanaka *et al.*, 1995). Even though Fas is expressed in tissues outside of the lymphoid system, defects in the Fas-FasL system appear to primarily affect the immune system (Krammer *et al.*, 1994).

Fas-mediated apoptosis can be induced by binding of the Fas receptor to the soluble or membrane-bound FasL, or by crosslinking the Fas receptor with either anti-Fas or anti-FasL monoclonal antibodies (mAbs). Fas expression alone is not sufficient to induce apoptosis and signal transduction through Fas is thought to require receptor trimerization (Dhein *et al.*, 1992). The exact nature of the biochemical pathway(s) that Fas signals through have not been elucidated, although a number of proteins that interact with the cytoplasmic domain of Fas have been identified using the yeast two-hybrid system (Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995).

The roles of Fas and FasL in apoptosis suggested that perhaps the phenotypes of *lpr* and *gld* mice resulted from defects in thymic selection. Defective negative selection would release autoreactive lymphocytes into the periphery where they induce autoimmunity and lymphoproliferation. Fas is constitutively expressed on the immature CD4+CD8+ thymocytes, the population that is susceptible to thymic selection. In 1995, it was demonstrated that treatment of CD4+CD8+ thymocytes with anti-Fas mAbs resulted in apoptosis both *in vitro* and *in vivo* (Ogasawara *et al.*, 1995). However, all evidence to date suggests that thymic selection is intact in *lpr* and *gld* mutants (Herron *et al.*, 1993). The most convincing evidence comes from *lpr* mice expressing a transgene-derived TcR specific for a known peptide. In these experiments, injection of the peptide induced deletion of thymocytes but not of mature peripheral T cells (Singer and Abbas, 1994). Therefore, it is currently thought that Fas does not play an obligatory role in thymic selection. Because *lpr* mice do express very low levels of Fas, there was some question

as to whether there was enough wild-type Fas to maintain normal thymic selection in these mice. This possibility was conclusively excluded by the creation of Fas-null (Fas^{-/-}) mice by targeted deletion of the Fas gene (Adachi *et al.* 1995). Compared with *lpr* mutants, the Fas^{-/-} mice exhibited enhanced and accelerated lymphoproliferation. In addition, injection of the superantigen staphylococcal enterotoxin (SEB), which reacts with V β 8 TcR's, caused deletion of immature thymocytes but not of mature peripheral T cells in the Fas^{-/-} mice (Osborne, 1996). *In vitro* experiments also show that activated mature T cells from *lpr* mice are refractory to AICD induced by a number of stimuli that cause apoptosis in activated mature wild-type T cells (Gillette-Ferguson and Sidman, 1994). Therefore all evidence points to a primary role for Fas-FasL in the peripheral AICD of mature T cells.

Research Goals

The lymphoproliferative phenotype of *scurfy* mice is suggestive of a breakdown in central and/or peripheral immune tolerance. A defect in central tolerance would likely result in the escape of potentially autoreactive T cells into the periphery, where they would proliferate in response to chronic stimulation from self-antigens encountered there. Misguided help from autoreactive T cells could lead to the production of autoantibodies, which would be detectable in *sf* serum. However, Godfrey *et al.* (1991) did not detect anti-nuclear antibodies for ribonuclear proteins (RNP), double-stranded DNA (dsDNA), or the small ribonuclear protein antigens SM, Ro, and La in *sf* sera. On the other hand, a defect in a mechanism involved in maintaining peripheral tolerance could also result in

the accumulation of activated cells in the peripheral lymphoid organs. Therefore, the goal of this research was to examine the expression and function of the Fas-FasL apoptotic pathway in *scurfy* mice.

Chapter 2

Methods and Materials

Mice

The *scurfy* (*sf*) mutation is maintained on a noninbred stock (DG) by mating *scurfy* carrier females (X^+X^{sf}) to (C3Hf/Rl x 101/Rl)F1 or (101/Rl x C3Hf/Rl)F1 males. *Sparse-fur* (*spf*) is an X-linked mutation in the ornithine transcarbamylase gene, located approximately 2-4 centimorgans (cM) from *sf*. The *scurfy sparse-fur* (*sf spf*) double mutation is maintained on a noninbred background by mating *sf spf* carrier females to (C3Hf/Rl x 101/Rl)F1 or (101/Rl x C3Hf/Rl)F1 males. C3H-*lpr/lpr* (*lpr*) breeder pairs were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the Oak Ridge National Laboratory. All stocks were housed in a conventional environment and fed a standard laboratory diet (Purina 5001) and chlorinated water *ad libitum*.

Cell Preparations

Cell suspensions from thymi and lymph nodes (axillary, inguinal, and cervical) were prepared by grinding tissue through a sterile 70 μ m nylon cell strainer (Falcon) on ice, followed by washing with cold phosphate-buffered saline (PBS). Cells were then passed through a 26-gauge needle to obtain a single-cell suspension. Nucleated cells were quantified using a model Zf Coulter Counter (Coulter Electronic, Inc.) according to the manufacture's instructions.

Antibodies

All antibodies were purchased from Pharmingen (San Diego, CA) for flow cytometric analysis. Thymocytes were double stained with fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated primary antibodies to CD4, CD8, CD69 (Very Early Activation Antigen), CD44 (Pgp-1, Ly-24), and CD25 (IL-2 Receptor alpha chain, p55). Lymph node cells were double-stained with the following directly-conjugated primary antibodies to α/β -TcR, CD45R/B220, CD4, CD8, CD44, CD25, CD62L (L-selectin, LECAM-1, Ly-22, MEL-14), CD45RB, CD69, and Fas (CD95). Streptavidin-phycoerythrin was used as a second step for labelling all biotinylated primary antibodies.

Flow Cytometric Analysis

Aliquots of 1×10^6 cells in 200 μ L of FACS buffer (0.1% sodium azide and 0.5% bovine serum albumin in PBS) were added to 12x74mm polystyrene tubes (Falcon) on ice. Non-specific binding of immunoglobulins was blocked by addition of 1 μ L of rat anti-mouse antibody specific for the Fc gamma II/III receptor (CD16/CD32) (Pharmingen, San Diego, CA). Directly conjugated primary antibodies were added at 1 μ g/ 1×10^6 cells in 200 μ L FACS buffer and incubated on ice for 30 minutes. Cells were then washed in 2ml of FACS buffer. If the primary antibody was conjugated to biotin, 2 μ L of streptavidin-phycoerythrin was added, the cells stained for 30 minutes on ice and washed again. Cells were resuspended in 500 μ L of FACS buffer and immediately analyzed on a FACScan II flow cytometer (Becton-Dickinson). At least 10,000 cells were assayed for each sample. Dead cells and debris were eliminated by gating on forward and side light scatter patterns

during data analysis.

Lymphocyte Activation for FasL mRNA Expression Analysis

Splenocytes isolated from wild-type and *scurfy* mice were activated *in vitro* with ConA (Takahashi *et al.*, 1994). 2×10^6 cell/ml were cultured in 0.5ml RPMI 1640 Complete medium (containing 10% fetal calf serum (FCS), 50mM β -mercaptoethanol, 1% penicillin/streptomycin., and 1% L-glutamine) in the presence of 2.5 μ g/ml ConA. At 48 hours, cells were collected and RNA isolated for reverse-transcriptase polymerase chain reaction (RT-PCR) analysis as described below.

Reverse Transcriptase (RT)-PCR Analysis

Total RNA was prepared from lymph nodes and thymi using guanidine isothiocyanate as previously described (Chomczynski and Sacchi, 1987). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco, BRL) and random hexamer primers (Gibco, BRL). The resulting cDNA was PCR amplified for 30 cycles (94°C, 1min.; 56°C, 1min.; 72°C, 1min. 30sec.) using Fas and FasL gene specific primers synthesized from published sequences. The Fas primers were 5' GTA CTA ATA GCA TCT CCG AG 3' and 5' CAG GGT GCA GTT TGT TTC CA 3' amplifying a 284bp fragment (Watanabe-Fukunaga *et al.*, 1992). The FasL primers were 5' ATG CAG CAG CCC ATG A 3' and 5' CTC ACG GAG TTC TGC C 3' amplifying a 342bp fragment (Takahashi *et al.*, 1994). As an internal control, primers were designed to amplify the β -actin gene. The β -actin primers were 5' ATG GGT CAG AAG GAC

TCC TA 3' and 5' GGT GTA AAA CGC AGC TCA GT 3' (Alonso *et al.*, 1986). 20 μ L of the PCR reaction product was analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Northern Blot Analysis

Total RNA was prepared from lymph node, spleen, and thymus tissues using guanidine isothiocyanate as previously described (Chomczynski and Sacchi, 1987). Ten micrograms of total lymph node and splenic RNA and 5 μ g of total thymic RNA was separated on a 1.5% denaturing formaldehyde gel and blotted onto a nylon membrane (Duralon-UV membrane, Stratagene) using standard procedures (Ausubel *et al.*, 1988). The Fas probe was generated by PCR amplification (described above) and labeled with [γ -³²P]dCTP using random hexamer primers. Washing was done to a final stringency of 0.1 x SSCP, 0.1% SDS at 65°C. Filters were exposed to X-ray film with intensifying screens for 48 hours at -80°C.

***In Vitro* Induction of Apoptosis**

Lymph node cells or thymocytes were cultured at 2x10⁶ cell/ml in 24-well flat-bottomed plates. Cells were cultured in complete medium (RPMI 1640 Complete with 10% fetal calf serum) for 16 hours at 37°C in 5% CO₂/air (Adachi *et al.*, 1995). Cells were collected from individual wells and assayed for apoptosis by flow cytometry as described below.

***In Vivo* Injection of anti-CD3 Ascites and Staphylococcal Enterotoxin B**

Individual mice were injected intraperitoneally with 200 μ L of anti-CD3 ascites (50 μ g or 100 μ g diluted in PBS) or Staphylococcal Enterotoxin B (SEB) solution (Sigma Chemical; 20 μ g or 50 μ g diluted in PBS). Control mice were injected with 200 μ L of PBS only. At various times (10, 24, or 48 hours), lymph nodes and thymi were removed and analyzed for surface markers and DNA fragmentation by flow cytometry as described below (Tucek-Szabo *et al.*, 1996).

***In Vivo* Injection of anti-Fas mAbs**

One hundred micrograms of purified anti-Fas mAb (Jo2; Phamingen, San Diego, CA) diluted in 200 μ L PBS was injected intraperitoneally into day 18 wild-type and *scurfy* mice. Thymi and lymph nodes were removed 4.5 hours after injection and assayed for DNA fragmentation by gel electrophoresis and flow cytometry as described below (Ogasawara *et al.*, 1995).

Gel Electrophoresis Analysis of DNA

The protocol for DNA analysis of apoptotic cells was similar to that previously described with modifications (Migliorati *et al.*, 1991). Briefly, 4×10^6 cells were dissolved in 100 μ L hypotonic lysing buffer (100mM NaCl, 10mM Tris, 1mM EDTA, 1% SDS, 200 μ g/ml proteinase K, pH 7.5) and incubated 30 minutes to overnight at 37°C. Samples were extracted with phenol and chloroform (1:1, v/v) and then with chloroform only. 100 μ g/ml RNase A was added to each sample, followed by incubation at 37°C for 30 minutes, and

phenol/chloroform extraction. The DNA was precipitated overnight at -20°C in 1/10 volume of 0.3M sodium acetate and 2.5 volumes of 100% ethanol and recovered by centrifugation. The DNA pellets were dissolved in TE buffer (10mM Tris-HCl, pH 8.0, and 1mM EDTA). 20µL were loaded onto a 2% agarose gel and visualized by ethidium bromide staining.

Quantitation of Apoptosis by Flow Cytometry

The procedure used to quantify apoptotic cells by flow cytometry was basically as described (Telford *et al.*, 1991). Briefly, 2×10^6 cells were washed in 1ml Hank's balanced salt solution (HBSS) and pelleted by centrifugation. The cells were fixed by rapid resuspension in 2ml of ice cold 70% ethanol and incubated for a minimum of 30 minutes at 4°C. Cells were pelleted by centrifugation and resuspended in 1 ml HBSS. After a 20 minute incubation at 37°C, the cells were washed in 1ml PBS and resuspended in 1ml of DNA Staining Reagent (PBS, pH 7.4, containing 0.1% Triton X-100, 0.1mM EDTA, pH 7.4, 0.05mg/ml RNase A, 50ug/ml propidium iodide). For immediate analysis, the cells were stained in DNA Staining Reagent for 1 hour at room temperature or were stored overnight in the dark at 4°C for analysis the next day. Cells were analyzed on a FACScan II flow cytometer (Becton-Dickinson), collecting on FSC log and FL2 linear 1. The threshold was set at FL2 of 30 and was set to exclude anything with less fluorescence than a chicken red blood cell (CRBC), (Fraker *et al.*, 1995). At least 10,000 events were analyzed for each sample. Apoptotic cells were defined as those cells with less fluorescence than G_0/G_1 but greater fluorescence than CRBCs.

Chapter 3

Results

Phenotype of Lymphocytes in Scurfy and Wild-type Mice

The differentiation and activation states of a lymphocyte are critical in determining its susceptibility to apoptosis. Therefore we examined the phenotypes of the lymphoid cells within the *scurfy* thymus and lymph nodes (inguinal, axillary, and cervical) by flow cytometric analysis of cell surface markers. *Scurfy* thymi were smaller in size compared to their wild-type littermates, as revealed by a 2-10 fold decrease in the total number of cells (Fig. 1, A*). Flow cytometric analysis revealed a dramatic decrease in the number of CD4+CD8+ double-positive T cells in the *scurfy* thymus (Ave. +/Y = $55.3\% \pm 18.4\%$; Ave. sf/Y = $9.2\% \pm 5.2\%$), with relative increases in the percentages of CD4-CD8-double-negative cells and CD4+CD8- or CD4-CD8+ single-positive cells (Fig. 2, A-B). This same thymocyte distribution is observed in mice treated with glucocorticoids, which results in the apoptosis of the CD4+CD8+ double-positive thymocytes (Gruber *et al.*, 1994). Therefore, rather than an intrinsic defect in thymocyte maturation, the abnormal thymocyte distribution observed in *scurfy* mice is likely to arise from the apoptosis of the CD4+CD8+ double-positive population in response to endogenously produced steroids. The expression of a number of maturation markers on *scurfy* and wild-type thymocytes were also analyzed (Fig. 3, A-B). *Scurfy* thymocytes exhibited staining profiles similar

* All figures and tables may be found in Appendix A.

to wild-type for CD44 and CD25, but had increased percentages of thymocytes expressing CD69 (%-CD4+CD69+: Ave. +/Y = 26.6% \pm 12.9%; Ave. sf/Y = 69.6% \pm 5.3%; %-CD8+CD69+: Ave. +/Y = 17.1% \pm 8.6%; Ave. sf/Y = 49.7% \pm 3.8%), (Fig. 3, A-B). CD69 is thought to play a role in thymocyte development, and is expressed on 30-60% of CD4+CD8- and CD4-CD8+ single-positive thymocytes (Yokoyama *et al.*, 1988; Swat *et al.*, 1993). Therefore the increased expression of CD69 on *scurfy* thymocytes is likely due to the increased relative percentages of CD4+CD8- and CD4-CD8+ single-positive thymocytes in *scurfy* thymi. However, it cannot be excluded that *scurfy* CD69-expressing thymocytes are activated. Anti-CD3 and PMA have been found to induce CD69 expression on thymocytes, and signalling through CD69 can induce thymocyte proliferation *in vitro* (Ziegler *et al.*, 1994; Yokoyama *et al.*, 1988).

Scurfy lymph nodes were greatly enlarged, and contained 2-4 times more cells than wild-type controls (Fig. 1, B). Within *scurfy* lymph nodes, the relative percentages of T cells were significantly decreased (+/Y = 77.3% \pm 8.2%; sf/Y = 68.3% \pm 4.3%; $p < .05$) with normal percentages of B cells (Fig. 4, A). However, because of the increase in absolute cell numbers, *scurfy* lymph nodes contain more absolute numbers of both T and B cells than wild-type lymph nodes. Within the T cell subset, *scurfy* lymph nodes contain significantly fewer CD4+ T cells (Ave. +/Y = 54.0% \pm 8.6%; Ave. sf/Y = 30.2% \pm 9.7%), with a slight increase in the relative percentage of CD8+ T cells (Fig. 4, B). These changes are most dramatically reflected in a decrease in the *scurfy* CD4/CD8 ratio (Ave. +/Y = 2.4 \pm 0.3; Ave. sf/Y = 1.1 \pm 0.3), (Fig. 4, C). *Scurfy* lymph node α/β -TcR+

cells were examined for changes in expression of various cell surface markers that are used to distinguish naive T cells from previously activated or memory T cells (Vitetta *et al.*, 1991). *Scurfy* lymph node T cells were consistently CD44^{high}, Mel-14^{low}, and CD45RB^{low}, a phenotype consistent with previous activation (Fig. 5, A-C). In contrast, wild-type lymph node T cells were CD44^{low}, Mel-14^{hi}, and CD45RB^{hi}, characteristic of naive or antigenically inexperienced T cells. (Fig. 5, A-C). These data suggested that a significant proportion of *scurfy* lymph node T cells have been previously activated (Budd *et al.*, 1987; Butterfield *et al.*, 1989; Birkeland *et al.*, 1989; Jung *et al.*, 1988; Lee *et al.*, 1990). A percentage of *scurfy* T cells were also CD69+ and CD25+ (Fig. 5, D-E). CD69 and CD25 are expressed early and transiently after T cell activation and their expression on *scurfy* T cells suggests that *in vivo* a majority of *scurfy* T cell have been recently activated (Yokoyama *et al.*, 1989; Sobel and Yokoyama, 1993; Ortega *et al.*, 1984; Moreau *et al.*, 1987). Both *scurfy* CD4+ and CD8+ T cell subsets contained activated cells (Table 1).

Fas and FasL mRNA Expression

To determine if *scurfy* lymphoid cells express *Fas* mRNA, RT-PCR was performed using *Fas* gene-specific primers on thymus and lymph node derived RNA. Both *scurfy* and wild-type thymi and lymph nodes expressed *Fas* (Fig. 6). Northern Blot analysis was used to quantitate *Fas* mRNA expression. *Scurfy* lymph nodes and spleen, but not thymus, appear to express higher levels of *Fas* mRNA than wild-type (Fig. 7).

RT-PCR was also performed using *FasL* gene-specific primers on unstimulated and *in vitro* ConA-activated spleen RNA. Both *scurfy* and wild-type activated splenocytes expressed *FasL* (Fig. 8). *FasL* mRNA was also detected in *scurfy* unstimulated spleen RNA, suggesting that *FasL* is constitutively expressed in *scurfy* spleens, but not wild-type spleens (Fig. 8).

Fas Cell Surface Expression

Next we examined the cell surface expression of Fas on lymph node cells in *scurfy* and wild-type mice by flow cytometry. The relative percentages of Fas+ α/β -TcR+, Fas+CD4+, and Fas+CD8+ T cells were similar in *scurfy* and wild-type (Fig. 9, B). However, *scurfy* lymph nodes contained significantly increased percentages of Fas+B220+ B cells (Fig. 9, B). All subsets in *scurfy* lymph nodes that were examined (α/β -TcR+, CD4+, CD8+, B220+) exhibited a significantly increased mean fluorescent intensity (MFI) of staining for Fas (Fig. 10, A-D), consistent with their activated phenotype. FasL cell surface expression could not be examined because antibodies were not commercially available. However, based on constitutive *FasL* mRNA expression in *scurfy* spleens, it is expected that the FasL protein would also be constitutively expressed on *scurfy* lymphoid cells.

***In Vitro* Induction of Apoptosis**

The expression of *Fas* mRNA and cell surface protein are necessary, but not sufficient, to mediate apoptosis. Therefore we examined the ability of *scurfy* lymph node cells to

undergo apoptosis. Freshly isolated wild-type and *scurfy* lymph node cells showed no differences in the percentages of cells undergoing apoptosis *ex vivo* as measured by flow cytometry (Fig. 11, A). However, *scurfy* lymph node cells showed significantly increased percentages of cells undergoing apoptosis after 16 hours of culture in complete medium compared to wild-type (Ave. +/Y = 34.5% \pm 10.7%; Ave. sf/Y = 51.8% \pm 8.3%), (Fig. 11, A). In addition, the percentages of freshly isolated *scurfy* thymocytes undergoing apoptosis were significantly greater than wild-type controls (Ave. +/Y = 0.9% \pm 0.5%; Ave. sf/Y = 2.7% \pm 2.1%), and after 16 hours in culture, an average of 65% of *scurfy* thymocytes had undergone apoptosis compared to 40% of wild-type thymocytes (Fig. 11, B).

***In Vivo* Induction of Apoptosis by Intraperitoneal Injection of anti-CD3 Ascites, Staphylococcal Enterotoxin B (SEB), or anti-Fas mAbs**

Although the above data suggest that *scurfy* thymocytes and lymph node cells do not have an intrinsic defect in the cellular apoptotic machinery, the question of their ability to undergo Fas-mediated apoptosis had not been addressed. Therefore, we injected anti-CD3 and SEB, substances that have been documented to induce apoptosis through a Fas-mediated mechanism into wild-type and *scurfy* mice (Tucek-Szabo *et al.*, 1996). However, injection of these compounds at all doses tested was lethal for *scurfy* mutants prior to the reported times at which apoptosis can be detected. (Table 2). Injection of anti-CD3 and SEB at all doses tested was never lethal for wild-type mice (Table 2). The rapid lethality of anti-CD3 and SEB in *scurfy* mice was likely due to toxic shock

syndrome (TSS) resulting from the activation of whole T cell subsets leading to massive cytokine secretion, multiple organ failure, and death (Chatila *et al.*, 1992).

We next intraperitoneally injected purified (no sodium azide, low endotoxin) anti-Fas mAb (clone Jo2) into *scurfy* and wild-type mice. 4.5 hours later, lymph nodes and thymi were harvested and analyzed for apoptosis by DNA gel electrophoresis (Fig. 12) and quantitated by flow cytometry (Fig. 13, A-B). Both wild-type and *scurfy* thymocytes were susceptible to Fas-mediated apoptosis as evidenced by DNA gel electrophoresis and flow cytometry (+/Y = 13.43%; *sf*/Y = 16.49%, 42.33%) Interestingly, a large percentage of *scurfy* lymph node cells, compared to wild-type, were susceptible to Fas-mediated apoptosis (+/Y = 5.61%; *sf*/Y = 48.03%, 21.90%).

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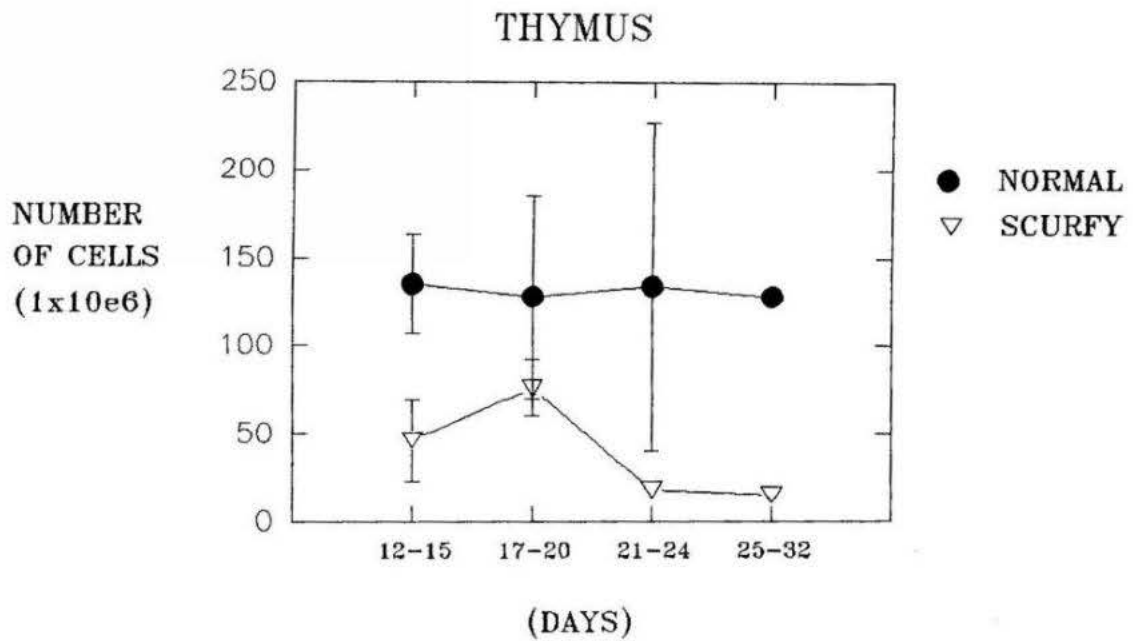
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Appendix Apoptosis

Figure 1. Absolute cell numbers in lymphoid organs. Single-cell suspensions were prepared and quantified from thymi and lymph nodes (cervical, inguinal, axillary) as described in Material and Methods. (A) *Scurfy* thymi contained 2-9 times fewer cells compared to age-matched wild-type thymi. (B) *Scurfy* lymph nodes contained 2-4 times more cells than age-matched wild-type littermates. Data represent the mean and standard error of the mean of 2-4 animals in each age group.

A.



B.

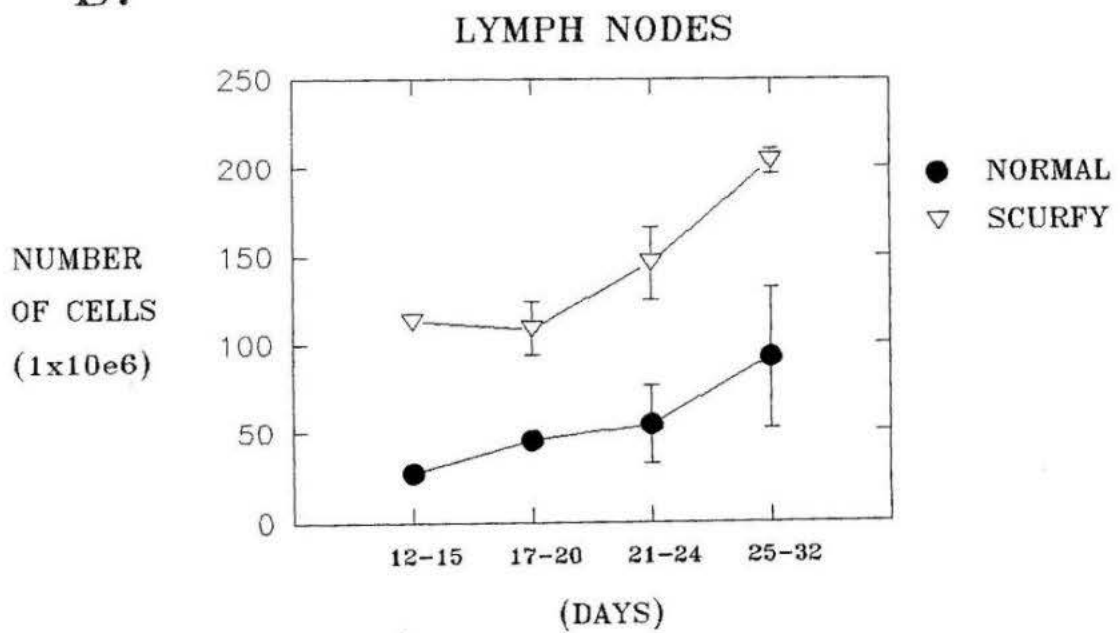
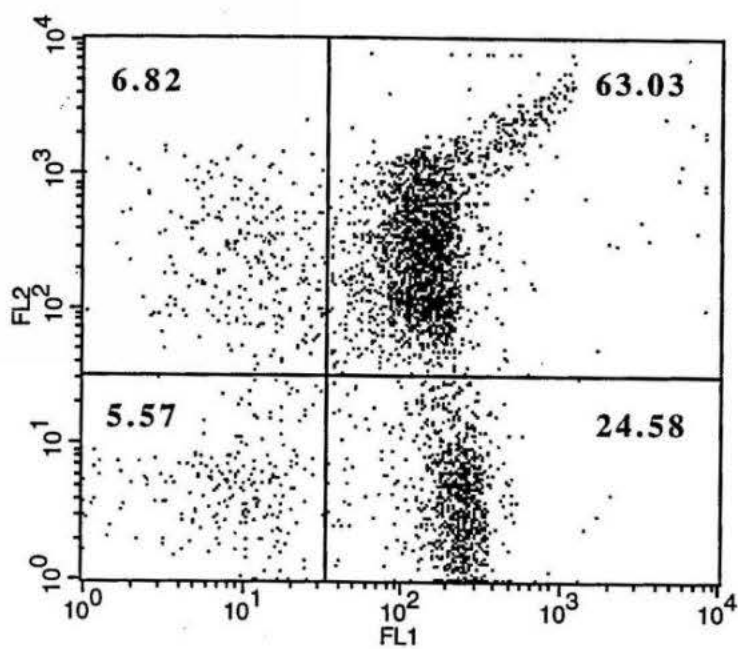


Figure 2. Flow cytometric analysis of thymocyte subsets. Thymocytes from day 18 wild-type (A) and *scurfy* (B) mice were stained for flow cytometric analysis as described in Materials and Methods. Dead cells and debris were eliminated on the basis of forward and side light scatter during analysis. Numbers are representative of the percentages of live cells per quadrant. Data are representative of three independent experiments (2 mice/experiment).

A.

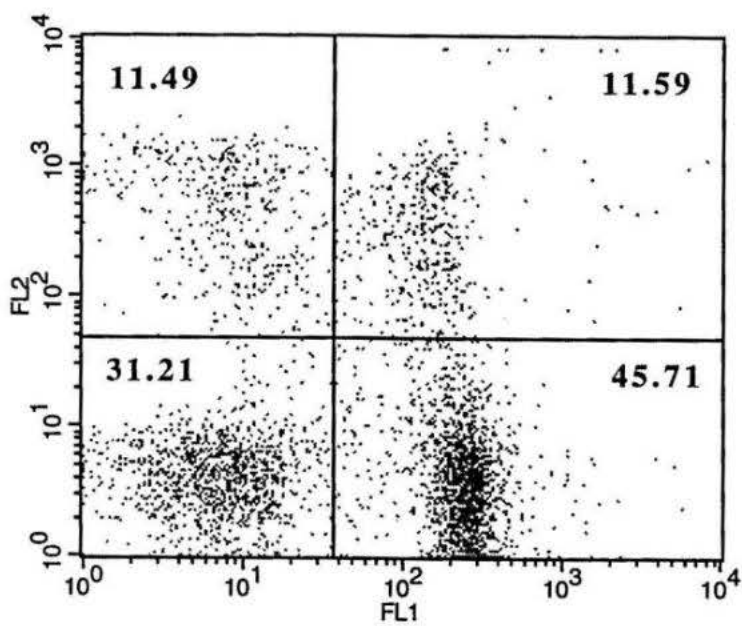
CD8



CD4

B.

CD8

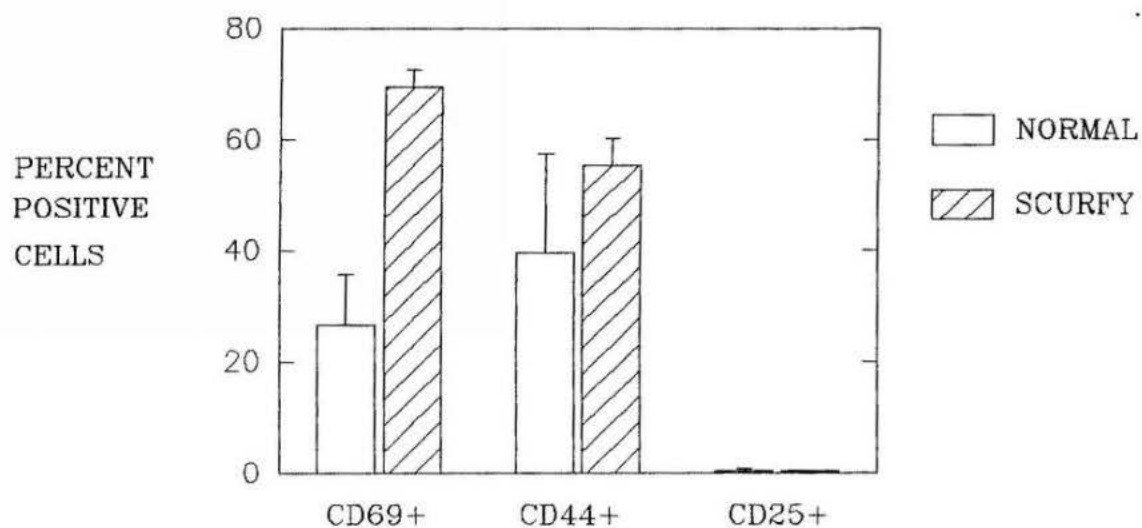


CD4

Figure 3. Flow cytometric analysis of thymocyte maturation markers. Thymocytes from day 18 wild-type and *scurfy* mice were stained for flow cytometric analysis as described in Materials and Methods. Thymocytes were gated on CD4+ or CD8+ T cells and dead cells and debris were eliminated on the basis of forward and side light scatter. (A) The percentage of CD4+ thymocytes that are CD69+ or CD44+ or CD25+. (B) The percentage of CD8+ thymocytes that are CD69+ or CD44+ or CD25+. Data represent the mean and standard error of the mean of three experiments (2 mice/experiment).

A.

CD4+ THYMOCYTES



B.

CD8+ THYMOCYTES

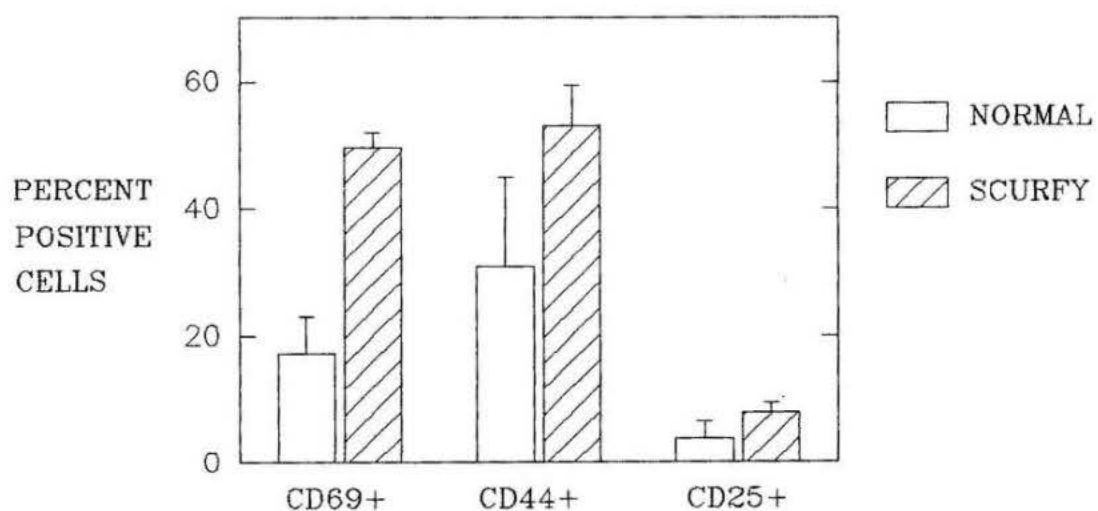
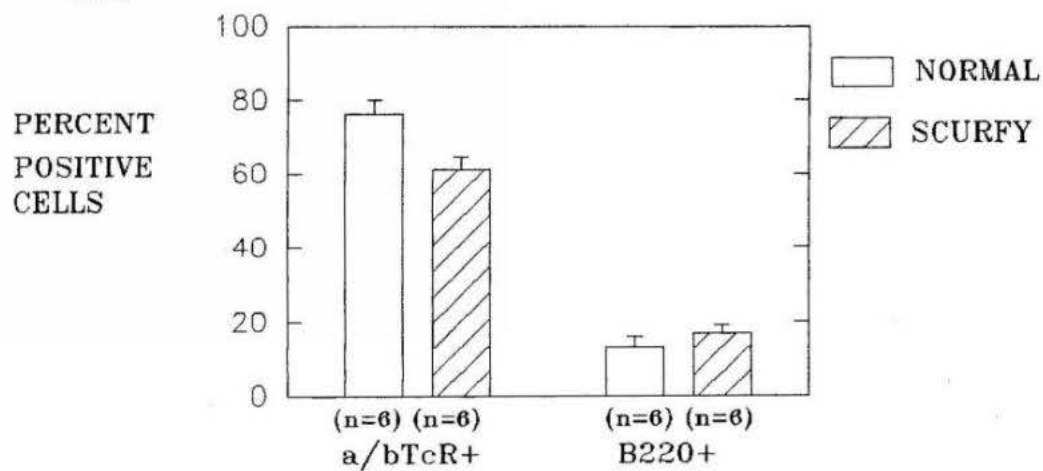
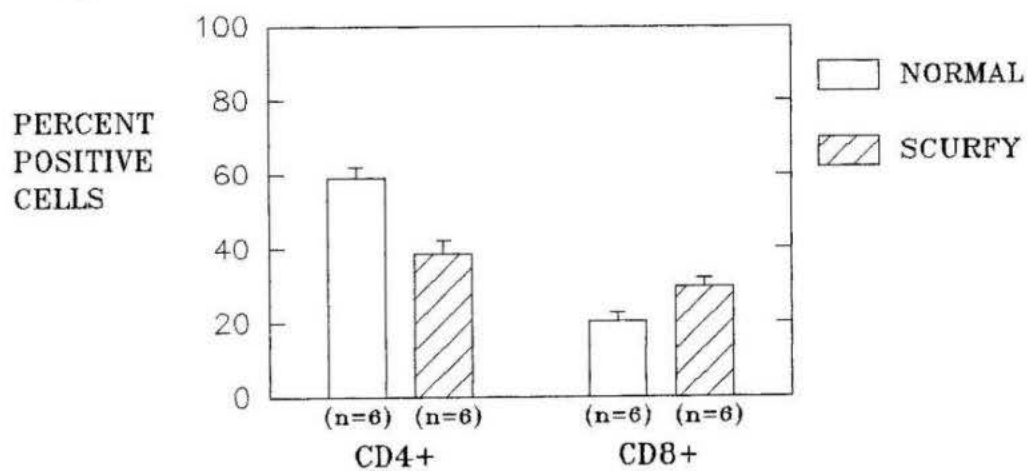


Figure 4. Flow cytometric analysis of lymph node subsets. Lymph node cells isolated from wild-type and *scurfy* mice (age 15-21 days) were stained for flow cytometric analysis as described in Materials and Methods. Dead cells and debris were eliminated on the basis of forward and side light scatter during analysis. (A) The percentages of α/β -TcR⁺ T cells and B220⁺ B cells; (B) the percentages of CD4⁺ and CD8⁺ T cells; (C) the CD4/CD8 ratio in the lymph nodes. Data represent the mean and standard error of the mean of at least three experiments (4 mice/experiment).

A.



B.



C.

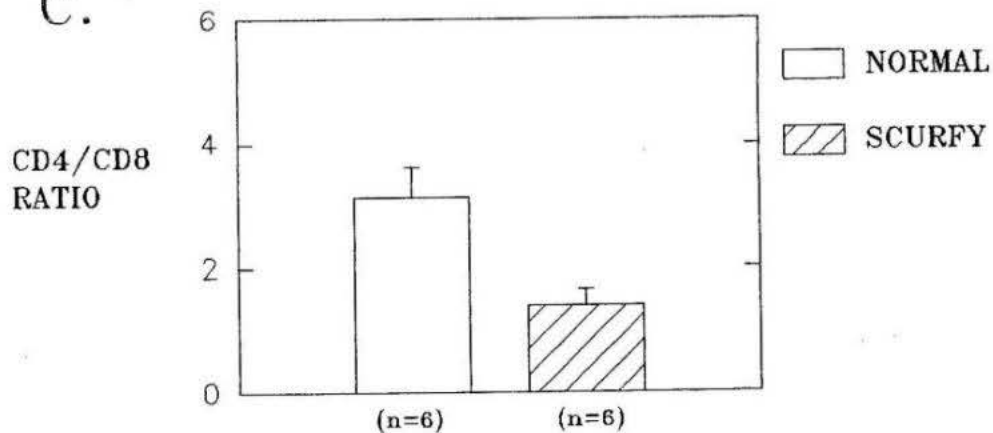
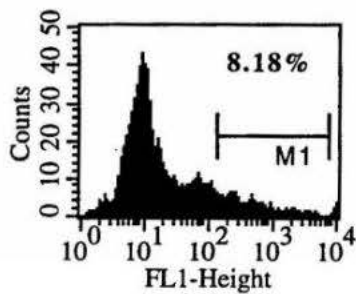


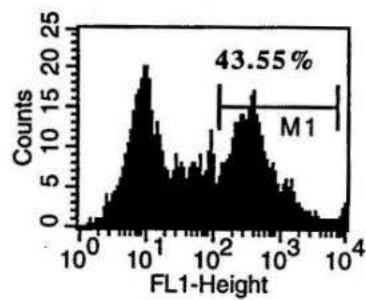
Figure 5. Altered expression of activation markers on *scurfy* lymph node T cells. Lymph node cells isolated from day 18 wild-type and *scurfy* mice were stained for flow cytometric analysis as described in Materials and Methods. Dead cells and debris were eliminated on the basis of forward and side light scatter during data analysis. The histograms represent the percentage of live α/β -TcR+ T cells that are (A) CD44^{high}; (B) Mel-14^{high} (C) CD45RB^{high}; (D) CD25+; and (E) CD69+. Data are representative of four independent experiments (2 mice/experiment).

A. normal lymph node



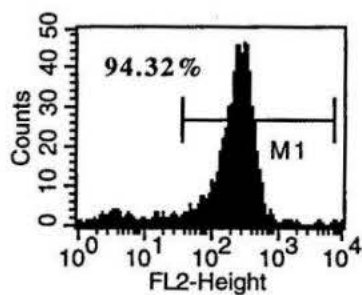
CD44

scurfy lymph node

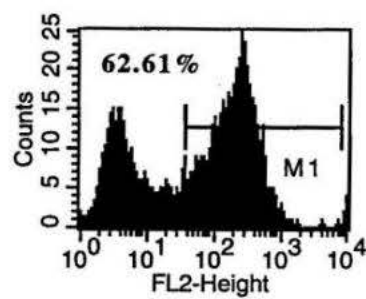


CD44

B.

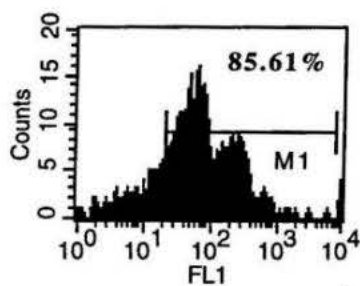


Mel-14

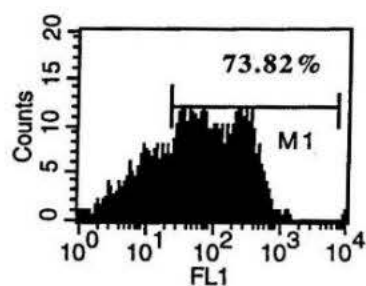


Mel-14

C.



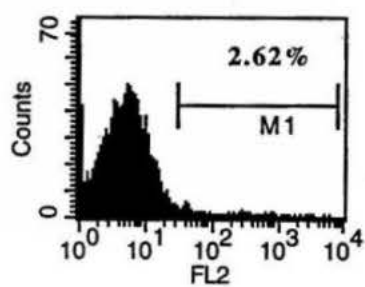
CD45RB



CD45RB

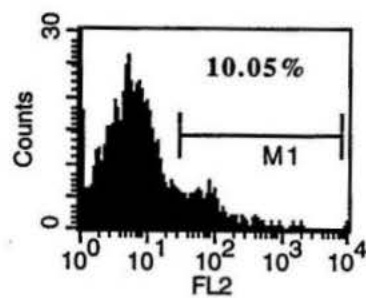
D.

normal lymph node



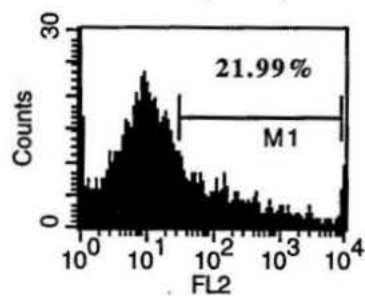
CD25 (IL-2Ra)

scurfy lymph node

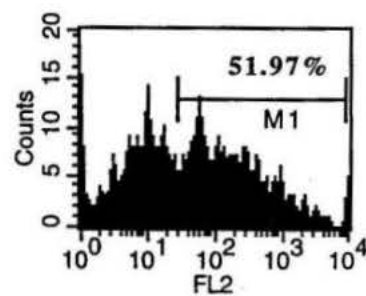


CD25 (IL-2Ra)

E.



CD69



CD69

Figure 6. RT-PCR analysis of *Fas* mRNA expression. RNA isolated from day 24 wild-type and *scurfy* mice was subjected to RT-PCR for *Fas* mRNA expression as described in Materials and Methods. A 284 basepair (bp) *Fas* band was amplified in lanes 2 (wild-type thymus), lane 4 (*scurfy* thymus), lane 7 (wild-type lymph node), and lane 9 (*scurfy* lymph node). Lanes 3, 5, 8, and 10 are negative control reactions (RT⁻ reactions). Lanes 1 and 6 are molecular weight standards (Φ x174/*Hae*III).

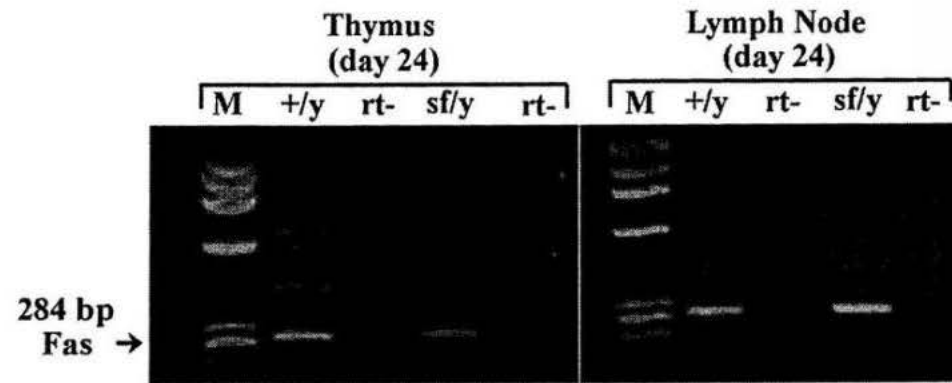


Figure 7. Northern blot analysis of *Fas* mRNA expression. Total RNA was isolated from day 24 wild-type and *scurfy* lymphoid organs. (A) The blot was probed with a radiolabeled PCR-generated *Fas* probe as described in Materials and Methods. (B) The ethidium bromide stained formaldehyde gel was used to standardize loading. Lanes 1, 4, 7 correspond to wild-type lymph node, spleen, and thymus RNA respectively. Lanes 2, 5, 8 correspond to *scurfy* lymph node, spleen, and thymus RNA respectively. Lanes 3, 6, 9 correspond to *lpr/lpr* lymph node, spleen, and thymus RNA respectively. The *Fas* mRNA corresponds to a 2.1 kilobase (kb) transcript (Watanabe-Fukunaga *et al.*, 1992).

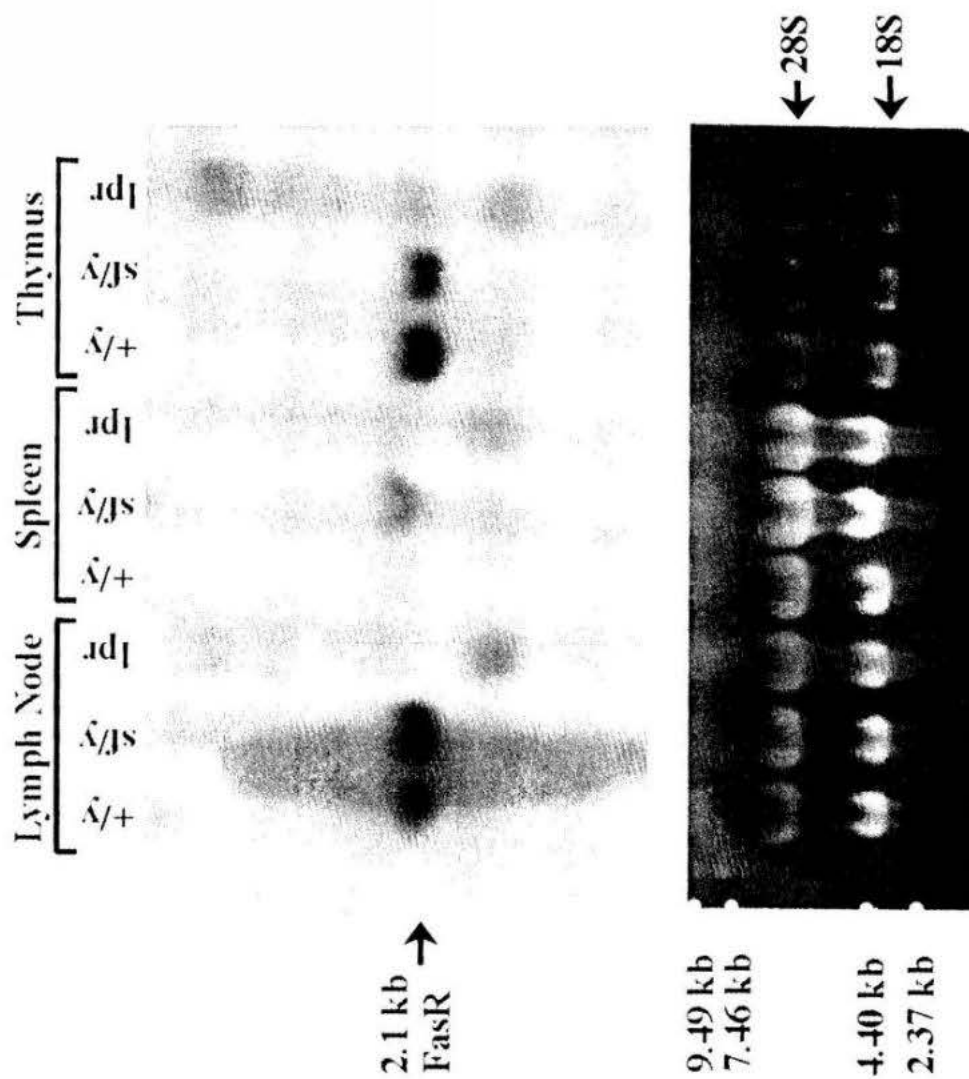


Figure 8. RT-PCR analysis of *FasL* mRNA expression. RNA was extracted from day 24 wild-type and *scurfy* unstimulated spleens and day 17 ConA-activated splenocytes and subjected to RT-PCR for *FasL* mRNA expression as described in Materials and Methods. A 340 bp fragment was amplified in lane 7 (wild-type activated spleen), lane 10 (*scurfy* unstimulated spleen), and lane 12 (*scurfy* activated spleen). Lanes 3, 5, 8, 11, and 13 are negative control reactions (RT⁻ reactions). Lane 4 is a β -actin positive control reaction for wild-type unstimulated spleen and amplified a 1kb fragment. Lanes 1, 6, and 9 are molecular weight standards (Φ x174/*Hae*III).

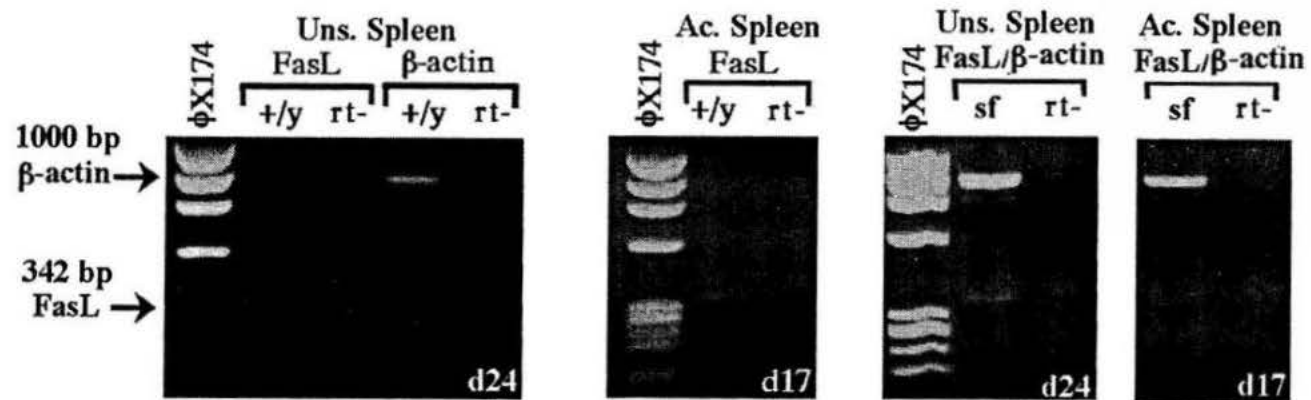
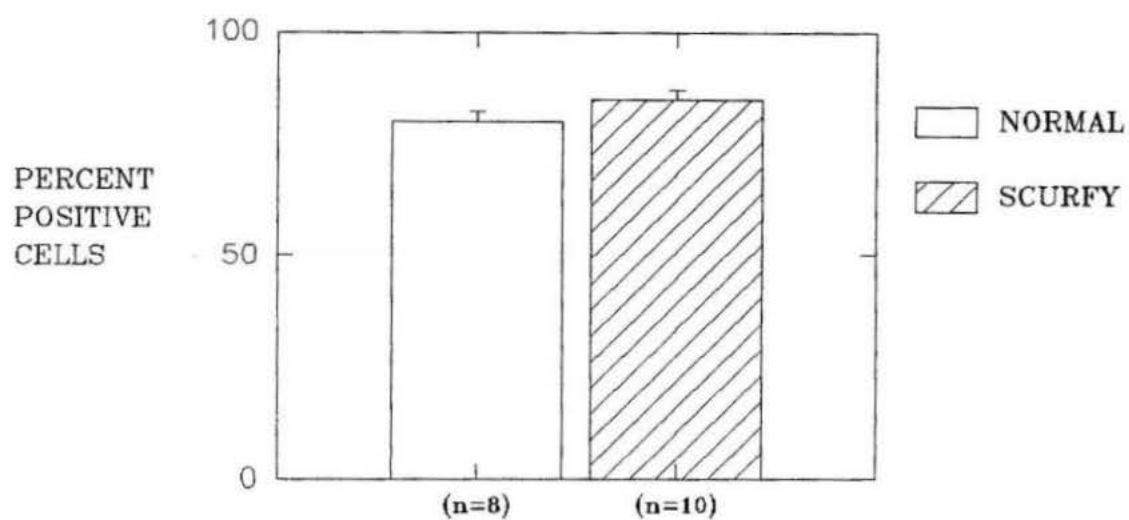


Figure 9. Flow cytometric analysis of Fas cell surface expression. Lymph node cells were stained for cell surface expression of Fas by flow cytometric analysis as described in Methods and Materials. (A) The percentage of Fas⁺ cells in wild-type and *scurfy* lymph nodes; (B) the percentage of CD4⁺Fas⁺, CD8⁺Fas⁺, and B220⁺Fas⁺ cells in lymph nodes. Live cells were discriminated by forward and side light scatter and gated on CD4⁺, CD8⁺ or B220⁺ cells. Data represent the mean and standard error of the mean of 7-13 experiments. The ages of the mice ranged from 15-21 days.

A.



B.

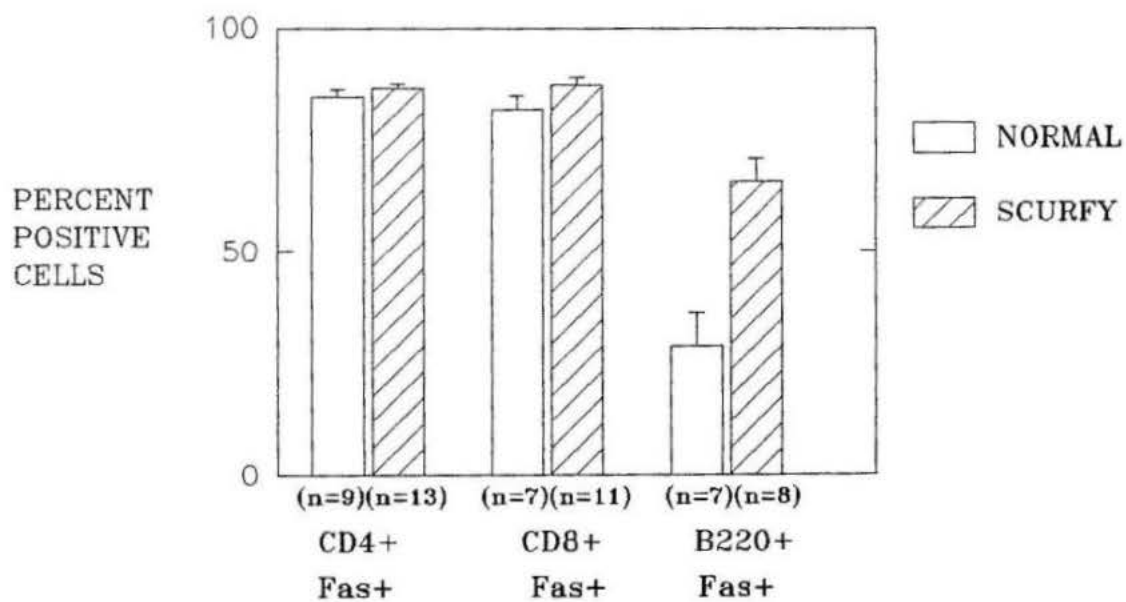
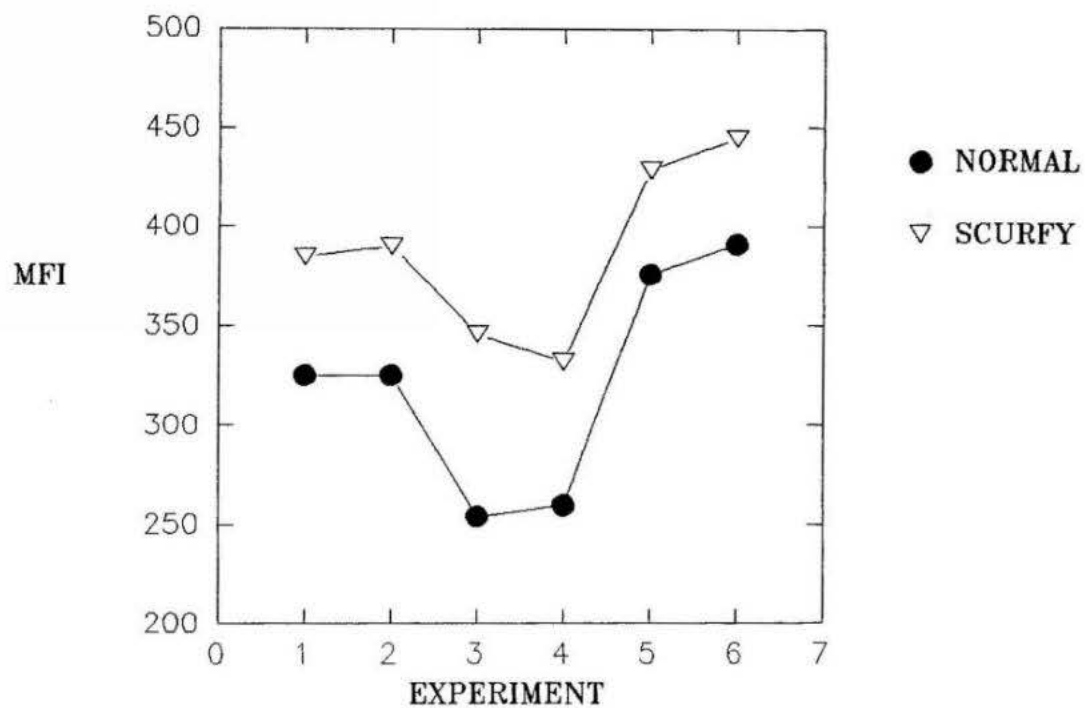


Figure 10. Mean fluorescent intensity of Fas cell surface expression. Lymph node cells were stained and analyzed as described in Figure 9. The mean fluorescent intensity (MFI) of (A) Fas⁺ only lymph node cells; (B) CD4⁺Fas⁺ T cells; (C) CD8⁺Fas⁺ T cells; and (D) B220⁺Fas⁺ B cells. Data represent the results of 4-7 independent experiments. The ages of the mice range from 18-21 days.

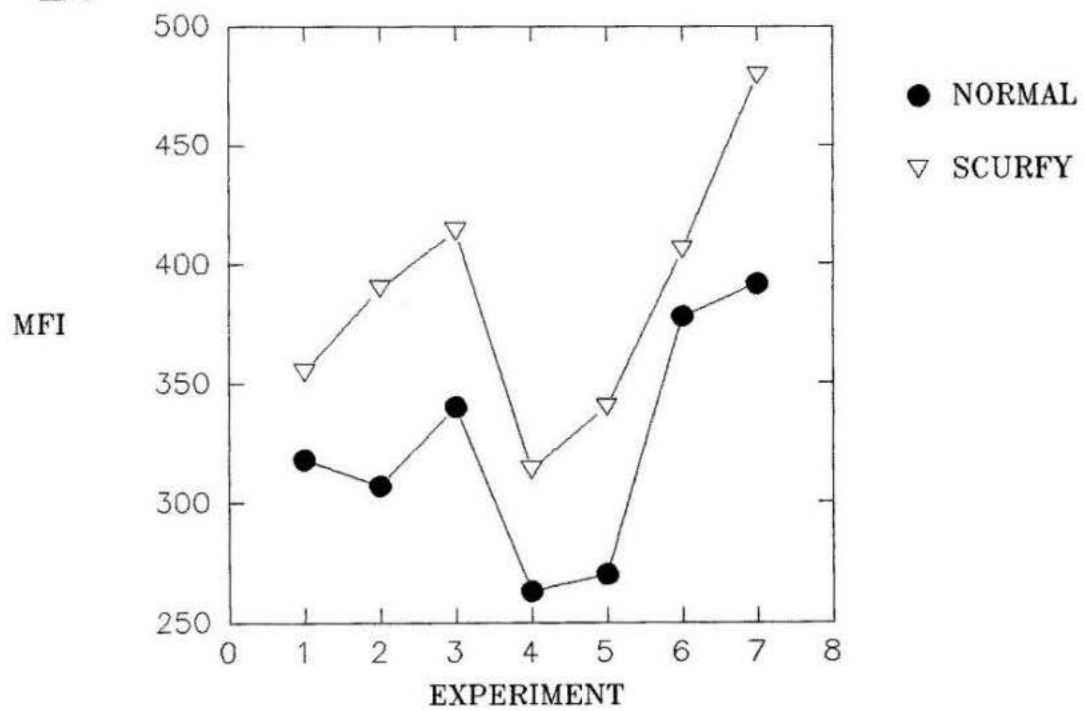
A.

FAS+ ONLY

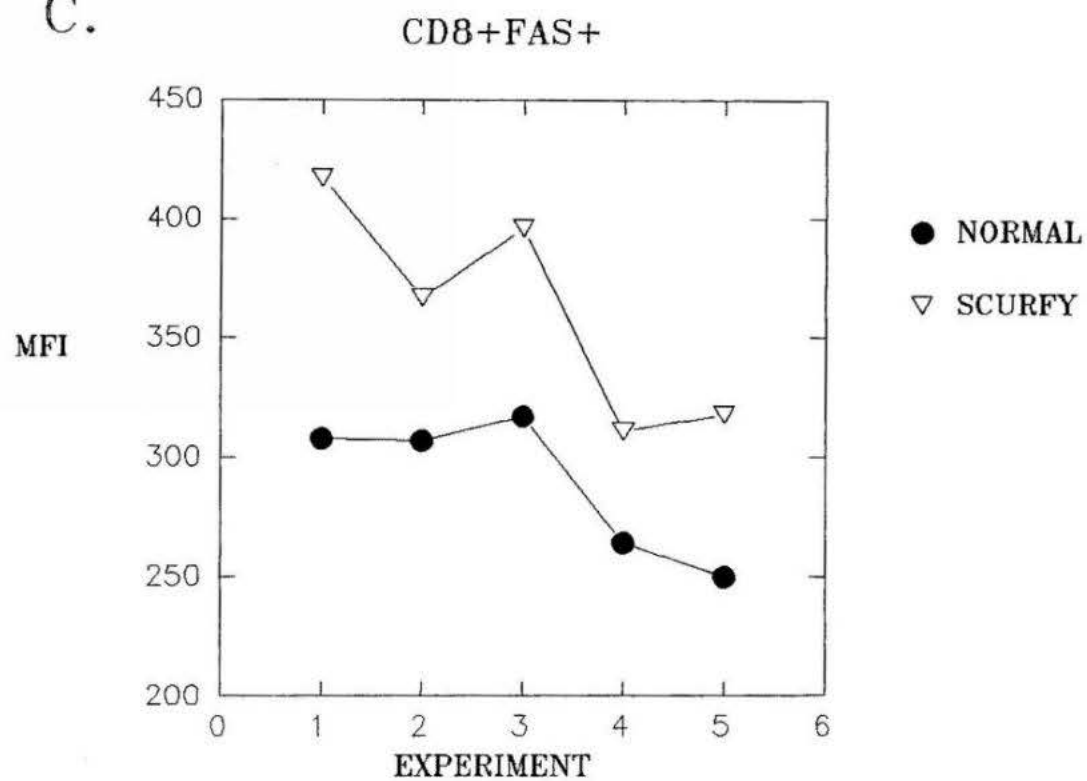


B.

CD4+FAS+



C.



D.

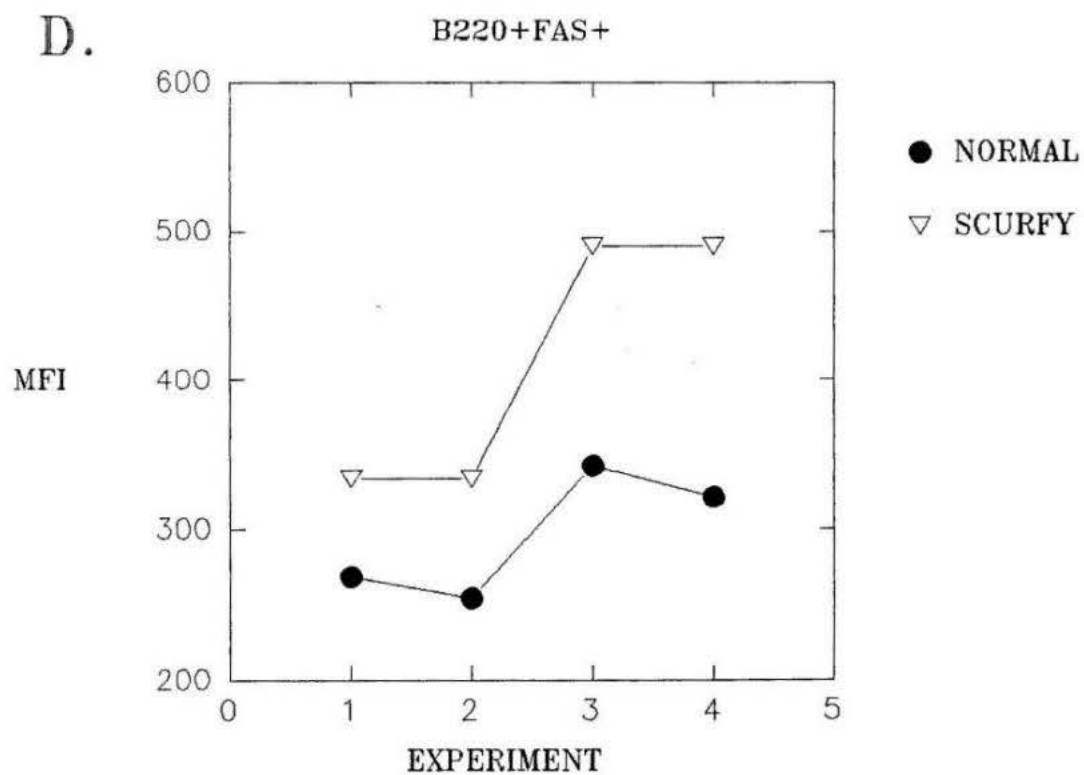
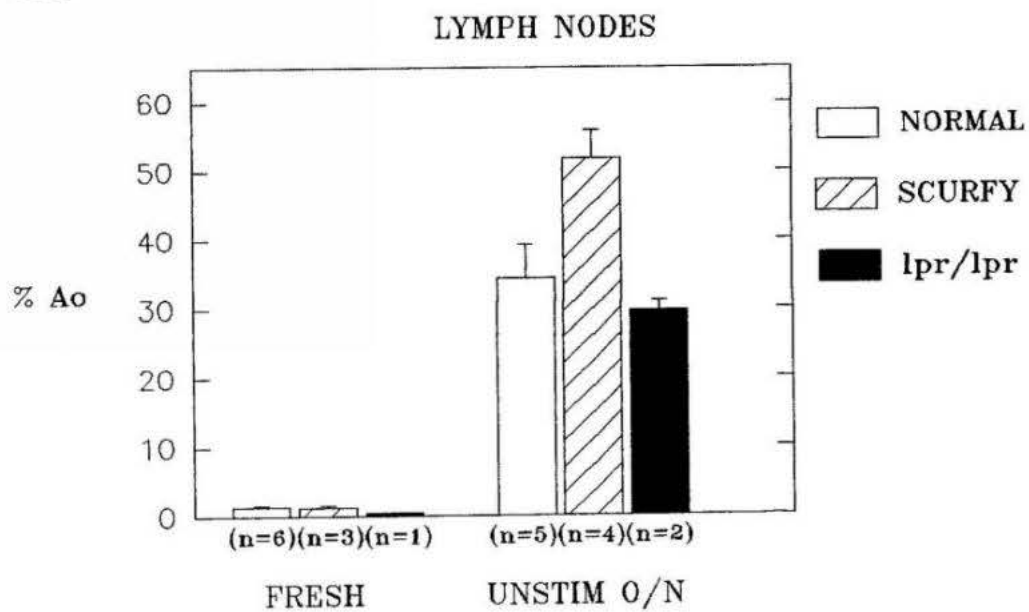


Figure 11. Spontaneous apoptosis of lymphocytes *ex vivo* and *in vitro*. Wild-type, *scurfy*, and *lpr/lpr* lymph node cells (**A**) and thymocytes (**B**) were removed and examined for *ex vivo* apoptosis (fresh) by flow cytometry. In addition, lymph node cells and thymocytes were cultured in RPMI 1640 Complete medium (10% FCS) as described in Materials and Methods. *Lpr/lpr* thymocytes and lymph node cells represent Fas negative controls. The percentage of cells undergoing apoptosis (% Ao) at 16 hours was quantitated by flow cytometry. Data represent the mean and standard error of the mean of 4-5 experiments.

A.



B.

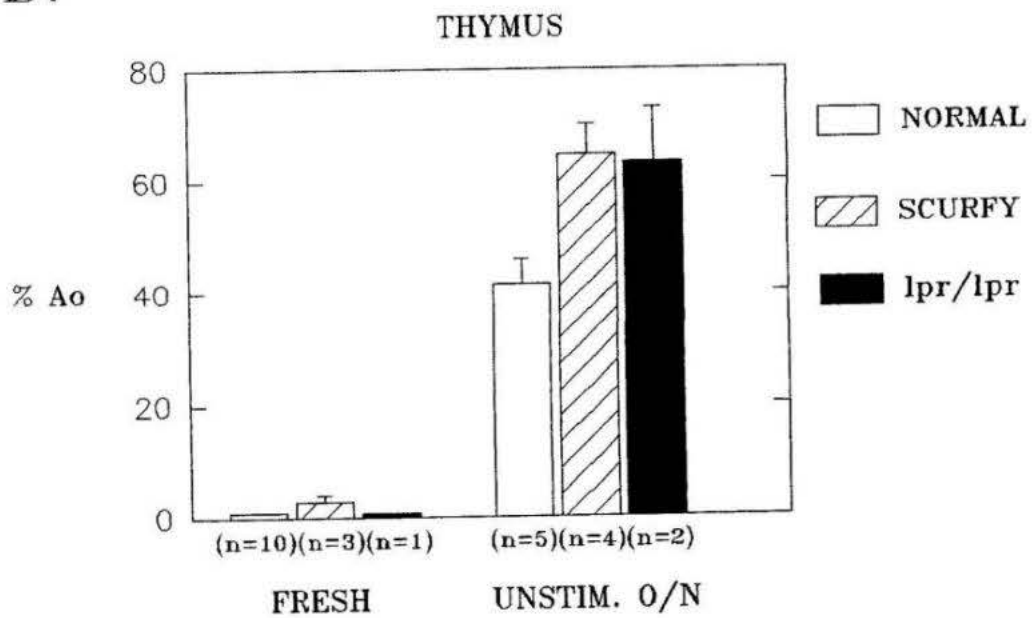


Figure 12. *In vivo* induction of Fas-mediated apoptosis. One hundred micrograms of purified anti-Fas mAb (clone Jo2) was injected into day 19 wild-type and *scurfy* mice. Lymph nodes, spleen, and thymus were removed 4.5 hours later and analyzed for DNA fragmentation by gel electrophoresis as described in Materials and Methods. DNA isolated from untreated wild-type and *scurfy* lymph nodes and thymus are represented in lanes 4, 6, 9, and 11 respectively. Lanes 5 (lymph nodes) and 10 (thymus) represent DNA isolated from wild-type mice 4.5 hours after injection with 100ug anti-Fas mAb. Lanes 7 and 8 (lymph nodes) and lanes 12 and 13 (thymus) represent DNA isolated from *scurfy* mice injected with 100ug anti-Fas mAbs. Lane 3 is a 100bp DNA marker and lane 2 is a dexamethasone-treated positive control for apoptosis.

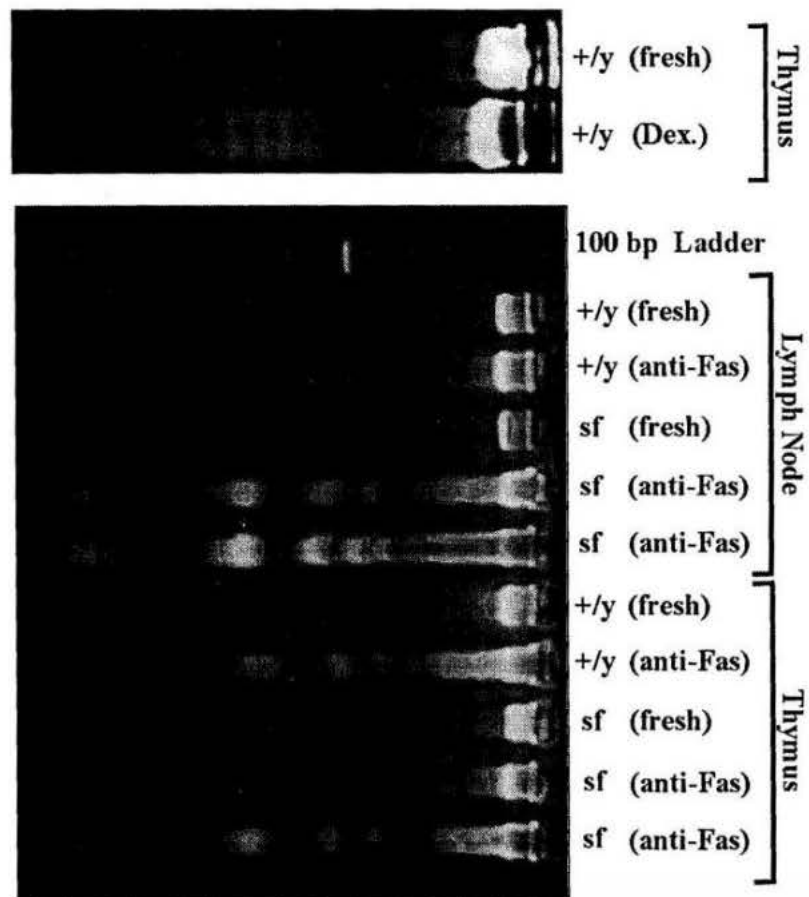
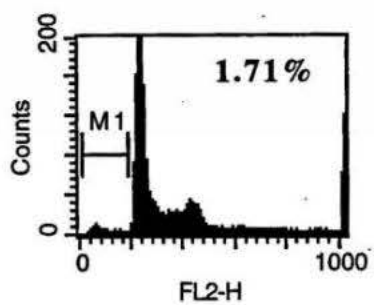


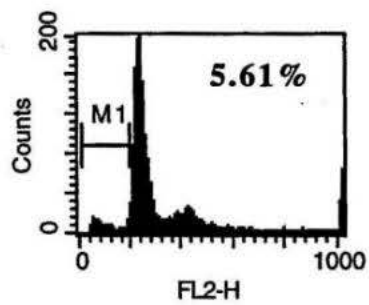
Figure 13. Quantitation by flow cytometry of apoptosis induced by injection of anti-Fas mAb. Anti-Fas mAb were injected as described in Figure 12. Staining of lymph node cells and thymocytes for flow cytometry were performed as described in Materials and Methods. M1 represents the subgenomic or apoptotic region of cells. The percentages represent the percent of cells that are in region M1. (A) represents the percentage of lymph node cells undergoing apoptosis and (B) represents the percentage of thymocytes undergoing apoptosis (n=2).

A.

WILD-TYPE

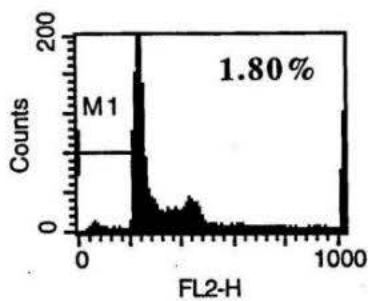


FRESH

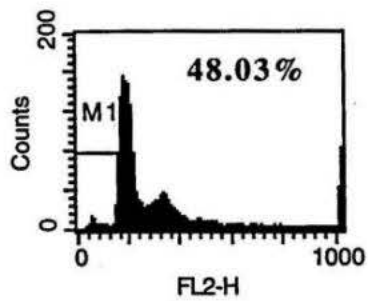


anti-FAS

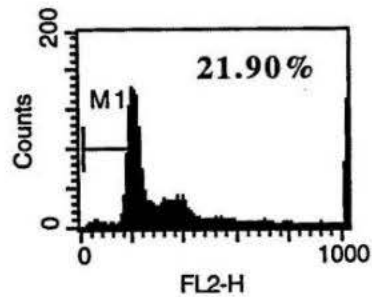
SCURFY



FRESH



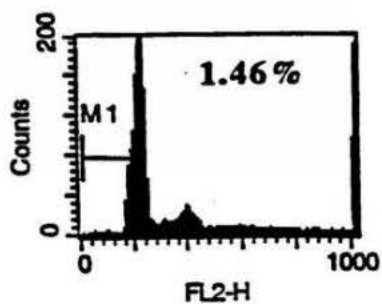
anti-FAS



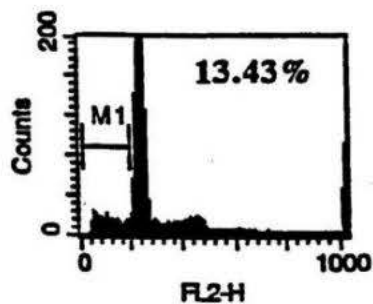
anti-FAS

B.

WILD-TYPE

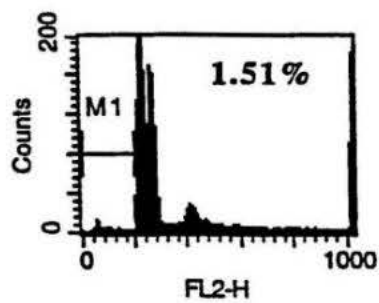


FRESH

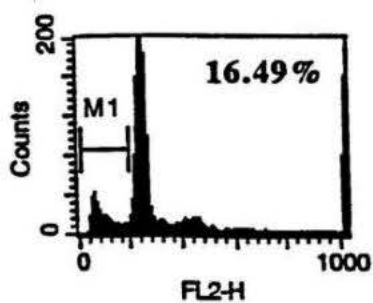


anti-FAS

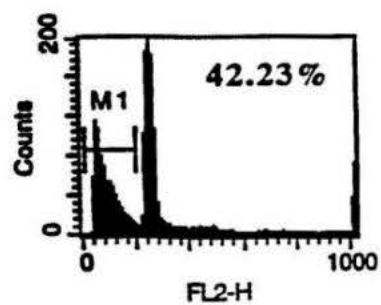
SCURFY



FRESH



anti-FAS



anti-FAS

Table 1: Activation markers on scurfy CD4+ and CD8+ lymph node T cell subsets

Mice	No.	% CD44 ⁺ ^{III} Cells		% CD69 ⁺ Cells		% CD45RB ⁺ ^{LO} Cells	
		CD4 ^a	CD8 ^b	CD4 ^a	CD8 ^b	CD4 ^a	CD8 ^b
+/Y	(1)	5.7	11.5	2.6	5.9	8.2	2.4
<i>sf/Y</i>	(3)	71.6 ± 9.4	39.5 ± 1.5	16.5 ± 3.2	16.0 ± 1.0	50.6 ± 2.9	5.1 ± 1.0

8

^a The percentage of CD4⁺ T cells from the lymph nodes of wild-type and *scurfy* mice which express CD44, CD45RB, and MEL-14 as analyzed by flow cytometry. Data represent the mean and standard error of the mean.

^b The percentage of CD8⁺ T cells from the lymph nodes of wild-type and *scurfy* mice which express CD44, CD45RB, and MEL-14 as analyzed by flow cytometry. Data represent the mean and standard error of the mean.

Table 2: Intraperitoneal injection of anti-CD3 ascites or Staphylococcal enterotoxin B

	age (days)	injection ^a	dose	hours	mortality (dead/total)
+/Y	(14; 21)	anti-CD3	100µg	48	0/3
<i>sf spf/Y</i>	(14)	anti-CD3	100µg	48	2/2
<i>sf/Y</i>	(21)	anti-CD3	100µg	48	1/1
+/Y	(12; 13)	anti-CD3	100µg	8-10	0/2
+/Y	(12; 13)	PBS		8-10	0/2
<i>sf spf/Y</i>	(12; 13)	anti-CD3	100µg	8-10	2/2
<i>sf spf/Y</i>	(12; 13)	PBS		8-10	0/2
+/Y	(13)	anti-CD3	50µg	48	0/1
+/Y	(13)	PBS		48	0/1
<i>sf spf/Y</i>	(13)	anti-CD3	50µg	48	2/2
<i>sf spf/Y</i>	(13)	PBS		48	0/1
+/Y	(11)	anti-CD3	50µg	24	0/1
+/Y	(11)	PBS		24	0/1
<i>sf spf/Y</i>	(11)	anti-CD3	50µg	24	1/1
<i>sf spf/Y</i>	(11)	PBS		24	0/1
+/Y	(20)	SEB	50µg	48	0/1
<i>sf/Y</i>	(20)	SEB	50µg	48	2/2
+/Y	(24)	SEB	20µg	24	0/1
<i>sf/Y</i>	(24)	SEB	20µg	24	0/1
Summary: (mortality)		anti-CD3	SEB	PBS	
+/Y		0/7	0/2	0/4	
<i>sf spf/Y</i>		7/7	nd ^b	0/4	
<i>sf/Y</i>		1/1	2/3	nd ^b	

^a Wild-type and *scurfy* or *scurfy sparse-fur* mice were injected intraperitoneally with various concentrations of anti-CD3 ascites (100µg, 50µg) and SEB (50µg, 20µg) diluted in 200µL PBS or PBS only.

^b Not done.

Part 3

Expression of Elements of the CD28/CTLA-4/B7 Pathway in *Scurfy* Mice

Chapter 1

Introduction

The Two Signal Model of T Cell Activation

The generation of an optimal immune response requires antigen-specific activation and clonal expansion of T cells into effector cells. The activation phase of this process requires two distinct signals. The first signal is mediated by ligation of the T cell receptor by antigen complexed to MHC molecules. The second, costimulatory signal is provided by ligands present on 'professional' antigen presenting cells (APC's) and is antigen-independent (Schwartz, 1990). Both signals are required for T cell activation, clonal expansion, and differentiation into effector cells. Antigenic stimulation in the absence of a costimulatory signal results in a functionally unresponsive state referred to as anergy (Harding *et al.*, 1992).

The B7-1/B7-2:CD28/CTLA-4 Costimulatory Pathway

The most well-documented costimulatory pathway involved in T cell activation is the B7-1/B7-2:CD28/cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) pathway (Norton *et al.*, 1992). This signalling pathway is extremely complicated as there are two T cell surface receptors and at least two ligands. Both receptors and ligands exhibit complex patterns of expression and there is evidence that the functional outcome of TcR ligation involves an integration of positive and negative signals from the TcR, CD28, and CTLA-4 (Chambers *et al.*, 1996).

CD28 and CTLA-4 T Cell Surface Receptors

CD28 and CTLA-4 are cell surface receptors belonging to the immunoglobulin superfamily (Gross *et al.*, 1990; Freeman *et al.*, 1992). It is thought that they arose by gene duplication as the *cd28* and *ctla-4* genes are linked on chromosome 2 in humans and chromosome 1 in mice and the protein receptors share a nucleotide sequence homology of about 75% (Dariavich *et al.*, 1988; Howard *et al.*, 1991). CD28 is constitutively expressed on virtually all murine T cells, and is upregulated upon activation (Gross *et al.*, 1992). The expression pattern of CTLA-4 is more complex. *Ctla-4* mRNA is not detectable in unstimulated T cells by Northern Blot analysis, but is readily detectable in activated T cells. Despite abundant mRNA expression, CTLA-4 cell surface expression on activated T cells is difficult to detect by flow cytometry, only reaching levels that are 3% of that observed for CD28 (Linsley *et al.*, 1992). Maximum CTLA-4 surface expression peaks between 24-48 hours after stimulation, with murine CD8⁺ T cells demonstrating higher levels than CD4⁺ T cells (Freeman *et al.*, 1992; Lindsten *et al.*, 1993; Linsley *et al.*, 1992). In 1995, it was shown that CTLA-4 contains an intracellular localization motif in its cytoplasmic tail which localizes the protein to the perinuclear Golgi and post-Golgi compartments (Leung *et al.*, 1995). Therefore, although CTLA-4 functions at the cell surface, it is mainly located intracellularly and continuously cycles to the surface (Linsley *et al.*, 1996).

The B7 Ligands

The CD28 and CTLA-4 receptors bind the same ligands, BB1/B7-1 (CD80) and B7-2

(CD86) expressed on APC's. Although CTLA-4 surface expression is minimal, it is the high avidity receptor for the B7 ligands, binding both B7-1 and B7-2 with a 20-50 times higher affinity than CD28 (Linsley *et al.*, 1991; Linsley *et al.*, 1994). The B7-1 glycoprotein was originally described as a B cell activation marker and its expression is restricted to 'professional' APC's such as activated B cells, dendritic cells, macrophages, and activated T cells (Larsen *et al.*, 1992; Azuma *et al.*, 1993; Hathcock *et al.*, 1994; Prabhu Das *et al.*, 1995). B7-2 is also expressed by 'professional' APC's, however, low levels of B7-2 have been detected on resting B cells as well. Both B7-1 and B7-2 expression are upregulated upon B cell activation (Freeman *et al.*, 1991; Lenschow *et al.*, 1993; Hathcock *et al.*, 1994). In addition, it was recently found that freshly isolated murine T cells express low levels of B7-2, but not B7-1, and both are upregulated upon T cell activation (Hathcock *et al.*, 1994; Inaba *et al.*, 1994). In addition to B and T cell activation, B7 expression is induced by signalling through the CD40/CD40L pathway, and influenced by a number of cytokines (Lenschow *et al.*, 1996a). The B7 ligands also exhibit differences in the kinetics of their expression. Regardless of the type of APC, B7-2 is expressed within 6 hours of stimulation, where as B7-1 is not detectable until 18-24 hours after stimulation (Lenschow *et al.*, 1993; Hathcock *et al.*, 1994). This led to the hypothesis that B7-1 was involved in the initiation of T cell responses, while B7-2 was important in sustaining the response (Boussiotis *et al.*, 1996). However, the functional outcome of differential B7 signaling appears more complicated than this, and some groups have suggested that these molecules can influence Th1/Th2 subset differentiation (Kuchroo *et al.*, 1995; Lenschow *et al.*, 1995a; Lenschow *et al.*, 1995b; Lenschow *et al.*,

1996b). Whether B7-1 and B7-2 induce distinct signalling pathways resulting in distinct functional outcomes is still unresolved.

CD28 Signalling

It has long been established that signaling through CD28 in conjunction with ligation of the TcR, provides a 'second' signal that is necessary for optimal T cell clonal expansion and differentiation into effector cells. CD28 signalling can prevent anergy largely in part because of its role in the initiation of transcription and stabilization of a number of cytokine mRNAs. Particularly important in preventing T cell anergy is CD28-dependent production of IL-2 and up-regulation of the IL-2R α , β , and γ chains (Lindsten *et al.*, 1989; Cerdan *et al.*, 1992). *In vitro* anergy caused by inadequate costimulation can be reversed by addition of exogenous IL-2 or other cytokines which signal through the common gamma chain of the IL-2R (Boussiotis *et al.*, 1994). Although the molecular basis for cytokine mRNA stabilization is not clear, it has been hypothesized that CD28 signalling interferes with the rapid turnover of cytokine mRNA's containing AU-rich instability sequences in their 3' untranslated regions (Schwartz, 1992).

More recent data in CD28 immunobiology has suggested that a primary function of CD28 may be to prevent apoptosis and enhance cell survival. Following TcR ligation and CD28 costimulation, the level of the cell survival protein, bcl-xl, increases on T cells (Chao *et al.*, 1995). Bcl-xl can heterodimerize with bax, and together they protect T cells from Fas-mediated apoptosis. The level of bcl-xl expression on T cells has been positively

correlated with resistance to Fas-mediated apoptosis (Boise *et al.*, 1995).

CTLA-4 Signalling

In contrast to the well documented role of CD28 in the costimulation of T cells, the role of CTLA-4 has long been controversial. Because of its homology to CD28 and the fact that it also binds the B7 ligands, with a higher affinity than CD28, it was hypothesized to play a similar, possibly synergistic role with CD28 in augmenting T cell proliferation in response to TcR ligation (Linsley *et al.*, 1991). Initial *in vitro* experiments supported this hypothesis as anti-CTLA-4 mAbs added to cultures stimulated with anti-CD3 mAb and anti-CD28 mAb resulted in an increase in T cell proliferation over cultures stimulated with anti-CD3 and anti-CD28 mAbs alone (Linsley *et al.*, 1992). However, further experiments using a CTLA-4-Ig fusion protein, consisting of the extracellular domain of CTLA-4 and the Fc portion of immunoglobulin G, and Fab fragments of the anti-CTLA-4 mAb suggested that blocking B7 interactions with CTLA-4 was not in fact sending a positive signal, but enhanced proliferation was a result of blocking a negative signal (Walunas *et al.*, 1994). In addition, crosslinking of CTLA-4 inhibited proliferation and IL-2 secretion by T cells stimulated with anti-CD3 and anti-CD28 mAbs (Krummel and Allison, 1995; Krummel and Allison, 1996). Although the *in vitro* data supported an inhibitory signalling role for CTLA-4 in T cell activation, it wasn't until 1995 when Waterhouse *et al.* created mice with a null mutation in CTLA-4 (CTLA-4^{-/-}) that a definitive role for CTLA-4 as a negative regulator of T cell activation was established (Waterhouse *et al.*, 1995).

CTLA-4^{-/-} Mice

Mice carrying a null mutation in CTLA-4 exhibit a massive lymphoproliferative disorder culminating in death at 2-3 weeks of age. The CTLA-4^{-/-} phenotype is characterized by the accumulation of activated T and B cells in the peripheral lymphoid organs of mutant mice and considerable infiltration of T cells into non-lymphoid organs mediating lethal tissue damage. Analysis of mutant thymi revealed increased proportions of CD4⁺ and CD8⁺ single-positive T cells, and a reduction in CD4⁺CD8⁺ double-positive T cells, resulting in a smaller thymus. Analysis of thymic maturation markers CD5, CD69, and CD44 suggested that thymic maturation was intact in CTLA-4^{-/-} mutants, and that the single-positive thymocytes were not activated. T cells isolated from the peripheral lymphoid organs of CTLA-4^{-/-} mutants showed expression patterns characteristic of previously activated T cells. Analysis of the relative percentages of various TcR V β elements indicated that the expansion of activated T cells in CTLA-4^{-/-} mice was polyclonal in nature. In support of their *in vivo* activated phenotype, CTLA-4^{-/-} T cells spontaneously proliferate in culture, and show increased proliferative responses compared to wild-type in response to stimulation through the TcR. These results suggest that in the absence of CTLA-4, T cells are activated, although the nature of the activating stimulus has not been resolved. A defect in apoptosis could also result in the accumulation of activated T cells in the periphery of CTLA-4^{-/-} mice. However, crosslinking of Fas in both wild-type and mutant T cells resulted in comparable levels of apoptosis. Based on this data, CTLA-4 has been suggested to be a negative regulator of T cell activation, and its absence results in dysregulated peripheral lymphocyte homeostasis (Waterhouse *et al.*,

1995; Tivol *et al.*, 1995).

Recent experiments to determine how CTLA-4 functions to negatively regulate T cell activation suggest that CTLA-4 inhibits T cell activation by blocking TcR/CD28-mediated IL-2 production and IL-2R expression, as well as upregulation of the T cell activation marker, CD69 (Krummel and Allison, 1996). Although it was reported that activated human T cells cultured in the presence of anti-CTLA-4 mAbs resulted in apoptosis, more recent reports suggest that CTLA-4 functions to down-regulate T cell activation in the absence of apoptosis (Gribben *et al.*, 1995; Krummel and Allison, 1996; Walunas *et al.*, 1996). This inhibition of T cell activation by CTLA-4 is accomplished by blocking T cell progression from the G₁ to the S phase of the cell cycle and is most pronounced 72 hours after initial T cell activation (Krummel and Allison, 1996; Walunas *et al.*, 1996).

Although apoptosis has long been recognized as the primary mechanism for maintaining both central and peripheral immune tolerance, the severe phenotype of CTLA-4 knockout mice suggests that other down-regulatory mechanisms may play an even more important role. Mice with defects in the Fas/FasL pathway, thought to be the main apoptotic pathway involved in peripheral tolerance, demonstrate progressive lymphadenopathy and autoimmunity which is generally not lethal until 6-8 months of age (Chu *et al.*, 1993; Takahashi *et al.*, 1994). In contrast, mice lacking CTLA-4 exhibit massive lymphoproliferation and autoimmune-like tissue damage that is lethal by 3-4 weeks of age (Waterhouse *et al.*, 1995; Tivol *et al.*, 1995). Taken together, this implies that

CD28/CTLA-4/B7 interactions may play a more significant role in the maintenance of peripheral lymphocyte homeostasis than Fas/FasL-mediated apoptosis.

Although the signalling pathways involved in CD28/CTLA-4/B7 signalling are complex and not fully understood, most evidence suggests that T cell activation involves the integration of positive signals mediated through the TcR and CD28, and negative signals transmitted through CTLA-4 (Chambers *et al.*, 1996). In fact, experiments involving CD28-deficient mice have implied that although CD28 is not necessary for CTLA-4 expression, CD28 signalling enhances CTLA-4 surface expression (Walunas *et al.*, 1994).

Maybe most importantly, this pathway offers new therapeutic approaches to transplantation, tumor immunity, and autoimmune disease. A number of experiments in which tumor cells were transfected with B7-1 or B7-2, resulted in potent and enhanced anti-tumor responses (Ramarathinam *et al.*, 1994; Townsend and Allison, 1993; Townsend *et al.*, 1994). In addition, blocking B7-signalling with CTLA-4Ig has extended, and in some cases prevented, transplant rejection in mice (Lenschow *et al.*, 1992). Lastly, CTLA-4Ig treatment has been demonstrated to significantly inhibit the onset of diabetes in the non-obese diabetic (NOD) autoimmune mouse model (Lenschow *et al.*, 1995a).

Research Goals

Scurfy mice exhibit a rapidly fatal lymphoproliferative disease with many phenotypic similarities to homozygous CTLA-4 knockout mice. Because *ctla-4* is an autosomal gene,

and *scurfy* is X-linked, they are definitely not the same gene. However, the phenotypic similarities between the two mutant mice suggests that they may involve overlapping signalling pathways. Therefore, the goal of this research was to examine the expression of *Ctla-4* mRNA and CTLA-4 protein expression in the *scurfy* mouse in an attempt to determine if the *scurfy* phenotype results from misexpression of CTLA-4.

Chapter 2

Methods and Materials

Mice

The *scurfy* mouse stocks were previously described (Part 2, Methods and Materials, pg. 19). All mice were used between 18 and 21 days of age.

Cell Preparations

Cell suspensions were prepared from lymph nodes (axillary, inguinal, and cervical) as previously described (Part 2, Methods and Materials, pg. 19)

Antibodies

All antibodies were purchased from Pharmingen (San Diego, CA). Non-specific binding of immunoglobulins was blocked by addition of CD16/CD32 (Fc gamma III/II Receptor) Fc Block to all cells to be stained for flow cytometric analysis. Lymph node cells were stained with directly-conjugated primary antibodies to $\alpha\beta$ -TcR, CD4, CD8, B220, TNP, CTLA-4, B7-1, B7-2, and CD28. Streptavidin-phycoerythrin was used as a secondary antibody to label the biotinylated anti-CD28 mAb. A hamster anti-TNP-PE IgG isotype control (Pharmingen, San Diego, CA) was used to subtract out background staining in all CTLA-4 samples.

Flow Cytometric Analysis

The procedure for surface staining of cells with mAbs for flow cytometric analysis was

described previously (Part 2, Methods and Materials, pg. 20). Twenty thousand events were collected for all CTLA-4 samples, and ten thousand events were collected for all other samples. Dead cells and debris were eliminated based on forward and side light scatter patterns during analysis.

Intracellular CTLA-4 Staining

The procedure used to stain cells for intracellular CTLA-4 expression was a modification of (Sander *et al.*, 1991; Prussin and Metcalfe, 1995). One million lymph node cells were suspended in 50 μ L of Staining Buffer (1ml FCS, 0.1g sodium azide in 100mls of PBS; pH 7.4-7.6) with optimal concentrations of FITC-labeled primary antibodies to $\alpha\beta$ -TcR, CD4, and CD8. The cells were stained on ice for 30 minutes. Cells were then washed with 2ml of Staining Buffer and pelleted by centrifugation. Cells were then fixed in 100 μ L of Fixation Buffer (4g of paraformaldehyde in 100mls PBS; pH 7.4-7.6) for 20 minutes on ice. After washing cells with Staining Buffer and pelleting by centrifugation, the cell pellet was resuspended in 50 μ L of Permeabilization Buffer (1ml FCS, 0.1g sodium azide, 0.1g saponin, 100mls PBS; pH 7.4-7.6). Optimum concentrations of either anti-TNP-PE or anti-CTLA-4-PE mAbs were added, and the cells incubated on ice. After 30 minutes, the cells were washed with Permeabilization Buffer, pelleted by centrifugation, resuspended in 1ml of Staining Buffer, and immediately analyzed on a FACScan II flow cytometer (Becton-Dickinson). Twenty thousand cells were collected for each sample. Dead cells and debris were eliminated on the basis of forward and side light scatter patterns during data analysis.

Reverse Transcriptase (RT)-PCR Analysis

The procedure used to isolate total RNA using guanidine isothiocyanate was previously described (Chomczynski and Sacchi, 1987). First-strand cDNA synthesis and PCR amplification were performed as previously described (Part 2, Methods and Materials, pg. 21) except the resulting cDNA was PCR amplified for 30 cycles (94°C, 1min.; 55°C, 1min.; 72°C, 1min. 30sec.) using murine *CTLA-4* gene specific primers synthesized from published sequences. The *CTLA-4* primers were 5' T ATT CAC ATG GAA AGC 3' and 5' ATG GCT TGT CTT GGA C 3' and amplified a 179bp fragment (Brunet *et al.*, 1987). As an internal control primers were designed to amplify the β -actin gene as described in Part 2, Methods and Materials, pg 21. 20uL of the PCR reaction product was analyzed on 1.5% agarose gel and visualized by ethidium bromide.

Proliferation Assays

Single-cell suspensions of lymph node cells and splenocytes were cultured in 96-well plates in 200 μ L of RPMI 1640 Complete media (10% FCS) alone or in the presence of Concanavalin A (ConA, 5 μ g/ml), or phorbol 12-myristate 13-acetate (PMA, 15ng/ml) and calcium ionophore A23617 (Ca²⁺, 250ng/ml) for 48 hours at 37°C. After 48 hours of culture, the cells were pulsed for 8 hours with 1 μ Ci of [³H]thymidine. Data represent the average of at least three wells per sample.

Chapter 3

Results

Ctla-4 mRNA Expression

Ctla-4 mRNA has not been detected in resting T cells by Northern Blot analysis, but is detected within 24 hours of *in vitro* stimulation (Freeman *et al.*, 1992). To determine if *scurfy* lymphoid cells express *Ctla-4* mRNA, we performed RT-PCR on unstimulated and ConA-activated spleen RNA using murine *Ctla-4* gene-specific primers. *Ctla-4* mRNA was not detected in wild-type unstimulated spleen, but was detectable after 48 hours of *in vitro* stimulation with ConA (Fig. 1, lanes 2 and 12). In contrast, *Ctla-4* mRNA was detectable in both unstimulated and ConA-stimulated *scurfy* spleen, suggesting that *Ctla-4* is constitutively expressed in *scurfy* spleens (Fig. 1, lanes 4, 7, and 9).

CTLA-4 Protein Expression

Although CTLA-4 functions at the cell surface, it is primarily expressed intracellularly in activated T cells (Leung *et al.*, 1995). To determine if *scurfy* lymph node T cells express CTLA-4, we analyzed non-permeabilized (surface) and permeabilized (intracellular) lymph node cells by flow cytometry for CTLA-4 expression. *Scurfy* lymph node α/β -TcR⁺ T cells expressed significantly ($p < .05$) more surface CTLA-4 and significantly ($p < .05$) more intracellular CTLA-4 than wild-type controls (Fig. 2; wild-

* All figures and tables may be found in Appendix B.

type = $1.8\% \pm 2.3\%$; *scurfy* = $8.9\% \pm 3.1\%$). To determine if one of the T cell subsets was responsible for the increased surface CTLA-4 expression on *scurfy* lymph node α/β -TcR⁺ T cells, we analyzed CTLA-4 surface expression on the CD4 and CD8 single-positive T cell populations in the lymph nodes. Although barely detectable on wild-type CD4⁺ T cells, CTLA-4 was increased 25-fold on *scurfy* CD4⁺ T cells (Fig. 3; wild-type = $0.2\% \pm 0.0$; *scurfy* = $5.1\% \pm 5.4\%$). This increase was not statistically significant ($p > .05$) because of one *scurfy* mouse expressing undetectable levels of surface CD4⁺CTLA-4⁺. However, because of the high affinity of CTLA-4 for its ligands, it is thought that even minimal levels of surface CTLA-4 are physiologically significant. Similar levels of surface CD8⁺CTLA-4⁺ were detectable in wild-type and *scurfy* lymph nodes (Fig. 3; wild-type = $15.7\% \pm 2.0\%$; *scurfy* = $19.1\% \pm 5.5\%$).

Expression of CD28, B7-1, and B7-2 by Flow Cytometry

In addition to CTLA-4 expression, we analyzed the cell surface expression of CD28, the constitutively expressed CTLA-4 homologue. Wild-type and *scurfy* α/β -TcR⁺ T cells demonstrated similar percentages and staining intensities for CD28 (Fig. 4; wild-type = $72.7\% \pm 0.3\%$; *scurfy* = $68.6\% \pm 8.9\%$). We also analyzed the surface expression of the shared CTLA-4 and CD28 ligands, B7-1 (CD80) and B7-2 (CD86). Compared to wild-type, an increased percentage of *scurfy* lymph node cells appeared to express B7-1, although this was not statistically significant because of small sample size (Fig. 5A and B; wild-type $10.1\% \pm 8.1\%$; *scurfy* = $24.7\% \pm 8.5\%$). To determine if this apparent increase in B7-1 expression was due to increased percentages of B220⁺ B cells expressing

B7-1, we double-stained lymph node cells for B220 and B7-1. By gating on B220+ B cells, *scurfy* exhibited significantly increased ($p<.05$) percentages of B7-1+ B cells (Fig 5C; wild-type = $29.7\% \pm 5.6\%$; *scurfy* = $49.8\% \pm 2.8\%$). Additionally, significantly increased ($p<.05$) percentages of *scurfy* lymph node cells expressed B7-2 compared to wild-type control (Fig. 6; wild-type = $22.4\% \pm 0.5\%$; *scurfy* = $34.0\% \pm 3.9\%$). Whether this increased expression of B7-2 was on B cells or activated T cells was not determined.

Mitogen Proliferation Assays

To examine the functional capabilities of *scurfy* T lymphocytes, we examined their *in vitro* spontaneous incorporation of tritium and their proliferative responses to ConA and PMA/Ca²⁺. ConA is a selective T cell mitogen which polyclonally activates and induces proliferation in T cells (Sharon, 1983). In contrast, PMA, which directly activates protein kinase C (PKC) and calcium ionophore A23187, which increases cytoplasmic free calcium, together activate T cells by bypassing cell surface specificity (Weiss *et al.*, 1984; Truneh *et al.*, 1985). Both *scurfy* lymph node cells and splenocytes demonstrated increased incorporation of tritium when cultured in medium alone, although because of variability between assays these values were not statistically significant (Fig. 7A; wild-type = 725.9 ± 625.1 ; *scurfy* = $1227.1 \pm 1,034$). *Scurfy* lymph node cells and splenocytes had decreased proliferative responses to the T cell mitogen, ConA, although only the later was statistically significant (Fig. 7B; wild-type = $95,924 \pm 85,899$; *scurfy* = $21,105 \pm 107,417$). In response to direct stimulation of PKC by PMA/Ca²⁺ both *scurfy* and wild-type lymph node cells and splenocytes incorporated similar levels of tritium (Fig. 7C).

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Appendix . Expression of the CD28/CTLA-4/B7 Pathway

Figure 1. RT-PCR analysis of *Ctla-4* mRNA expression. RNA was extracted from wild-type and *scurfy* unstimulated and ConA-activated spleens and subjected to RT-PCR for *Ctla-4* mRNA expression as described in Materials and Methods. A 180bp *Ctla-4* fragment was amplified in lane 4 (wild-type activated splenocytes) and 7 (*scurfy* unstimulated spleen), and lane 9 (*scurfy sparsefur* activated spleen). Lanes 3, 5, 8, and 10 are negative control reactions (RT⁻ reactions). Lane 2 is a β -actin control reaction for wild-type unstimulated spleen and amplifies a 1kb fragment. Lanes 1 and 6 are molecular weight standards (Φ x174/*Hae*III).

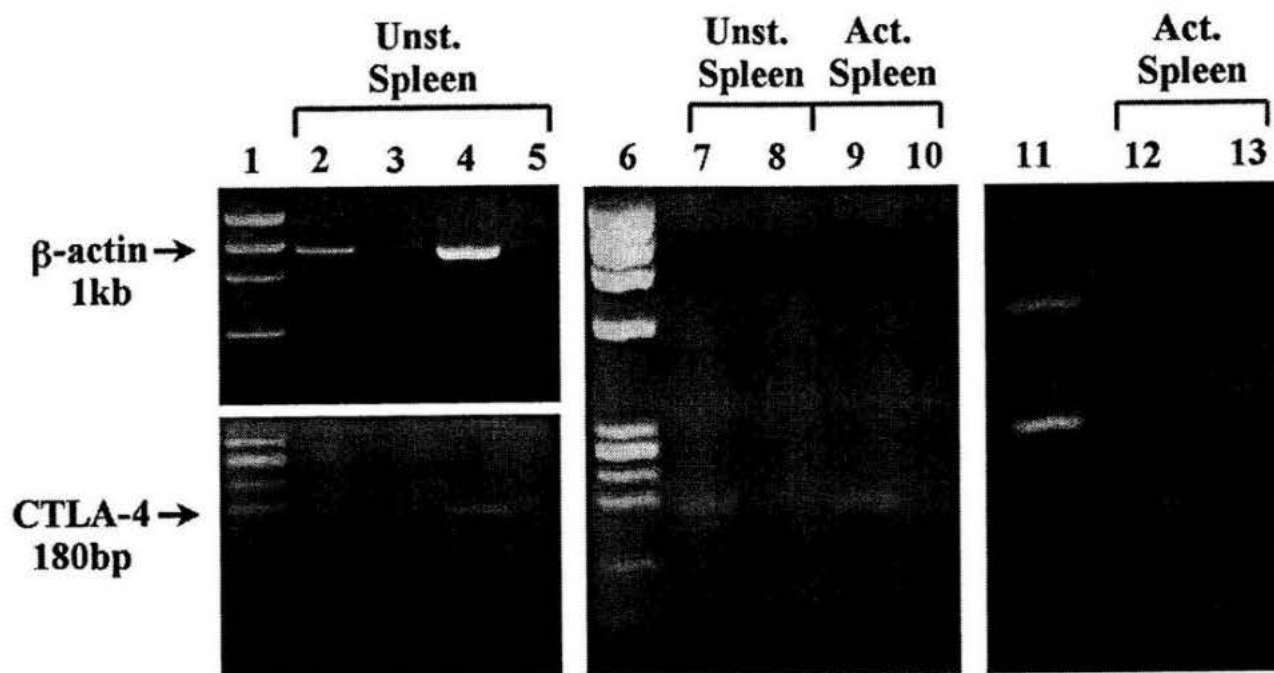
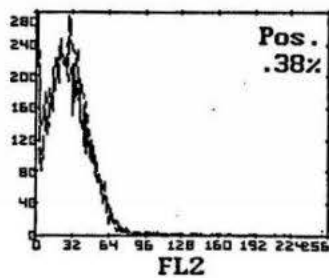


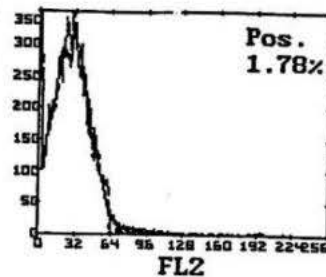
Figure 2. Flow cytometric analysis of CTLA-4 cell surface and intracellular expression. Lymph node cells from day 19 wild-type and *scurfy* mice were stained for cell surface and intracellular CTLA-4 expression by flow cytometric analysis as described in Methods and Materials. A representative sample of the data is shown in (A). Histograms were generated by gating on α/β -TcR⁺ T cells and analyzing CTLA-4 expression on this population. The percentage of positive cells was determined by subtraction of a negative isotype control and is represented by the shaded area in the histograms. Dead cells and debris were eliminated by gating on forward and side light scatter during data analysis. (B) Both surface and intracellular CTLA-4 expression are upregulated in *scurfy* mice (n=4) compared to wild-type (n=3).

A.

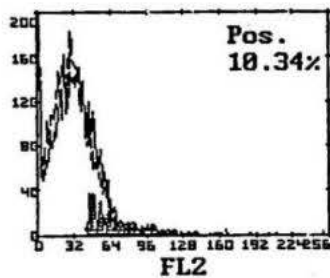
NORMAL SURFACE



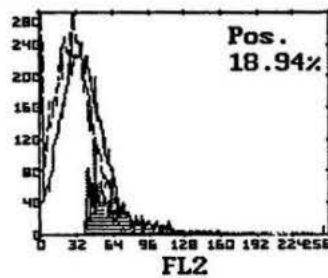
NORMAL PERMEABILIZED



SCURFY SURFACE



SCURFY PERMEABILIZED



B.

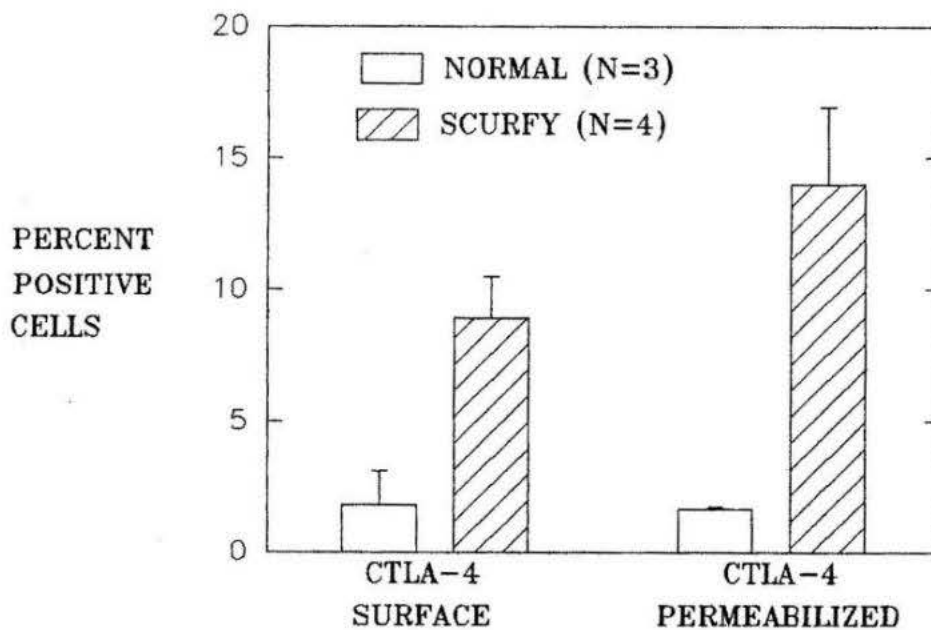
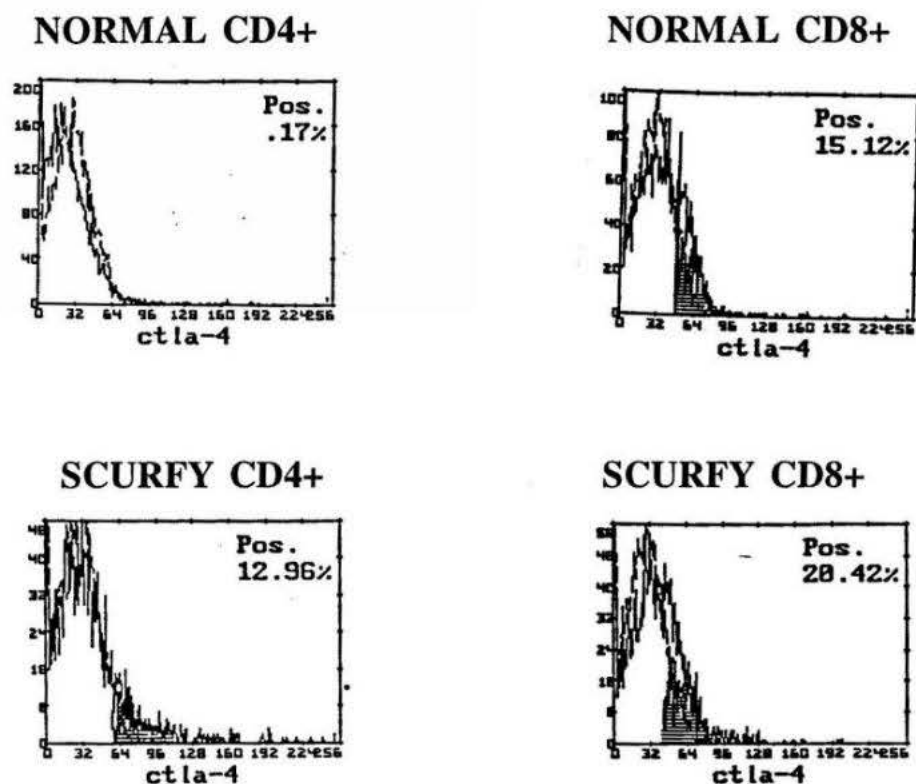


Figure 3. Flow cytometric analysis of cell surface CTLA-4 expression on lymph node T cell subsets. Lymph node cells from d19 wild-type and *scurfy* mice were double stained for CD4 or CD8 and CTLA-4 as described in Methods and Materials. A representative sample of the data is shown in (A). Histograms were generated by gating on CD4⁺ or CD8⁺ T cells and analyzing CTLA-4 expression on this population. The percentage of positive cells was determined by subtraction of a negative isotype control. Dead cells and debris were eliminated by gating on forward and side light scatter during analysis. (B) The upregulation of CTLA-4 in *scurfy* lymph node T cells (n=4) is primarily on the CD4⁺ T cell subset.

A.



B.

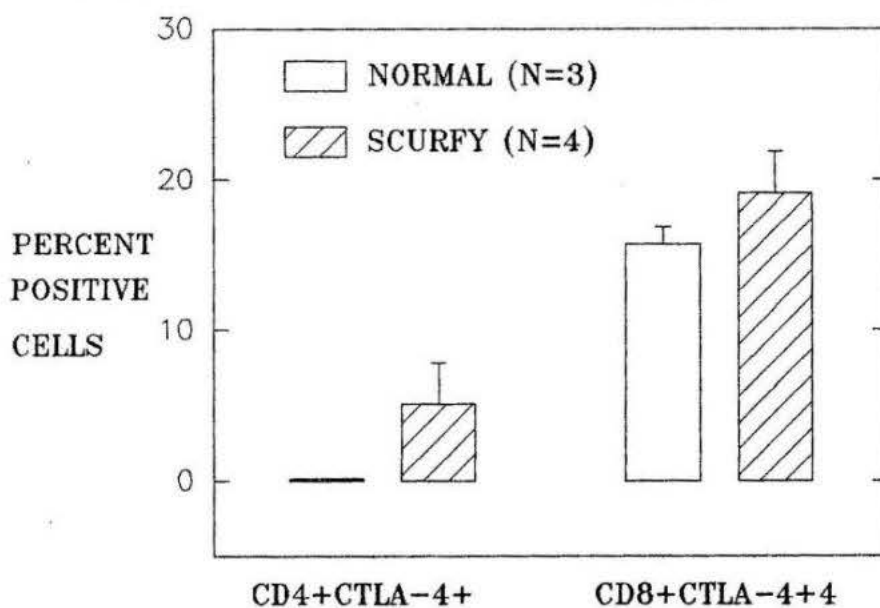
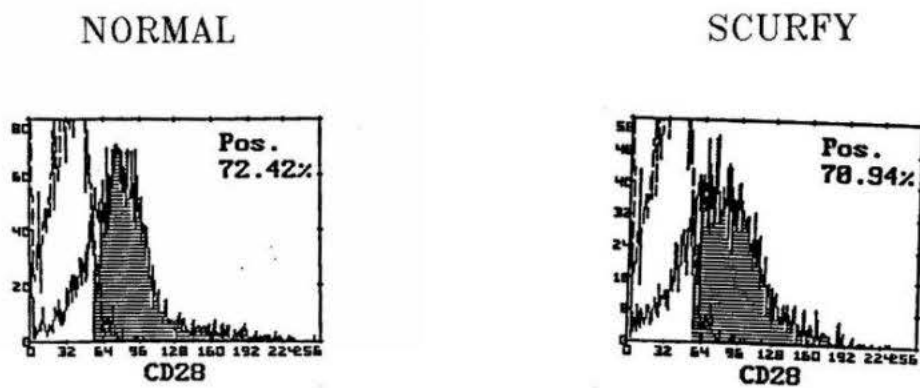


Figure 4. Flow cytometric analysis of CD28 cell surface expression. Lymph node cells from day 19 wild-type and *scurfy* mice were stained for cell surface expression of α/β -TcR and CD28 as described in Methods and Materials. A representative sample of the data is shown in (A). Histograms were generated by gating on α/β -TcR⁺ T cells and analyzing CD28 expression on this population. The percentage of positive cells was determined by subtraction of a negative isotype control. Dead cells and debris were eliminated by gating on forward and side light scatter during data analysis. (B) CD28 is expressed similarly on wild-type (n=3) and *scurfy* (n=3) lymph node T cells.

A.



B.

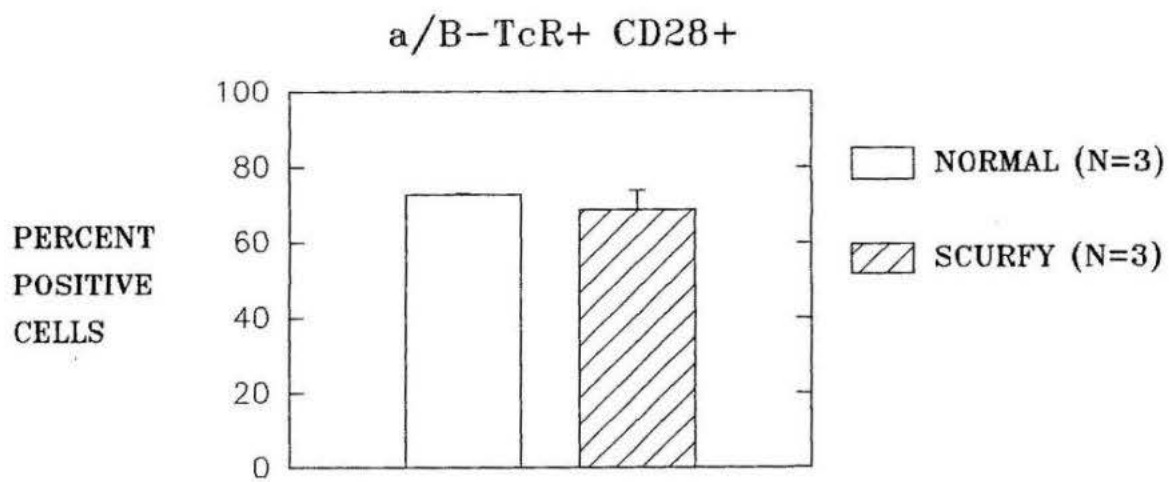
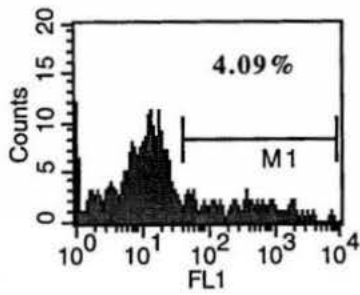


Figure 5. Flow cytometric analysis of B7-1 cell surface expression. Lymph node cells from day 19 wild-type and *scurfy* mice were stained for cell surface expression of B7-1 (CD80) as described in Methods and Materials. A representative sample of the data is shown in (A). Dead cells and debris were eliminated by gating on forward and side light scatter during data analysis. (B) The expression of B7-1 on whole wild-type and *scurfy* lymph node populations is not significantly different ($p>.05$). (C) But *scurfy* lymph nodes ($n=3$) have significantly increased ($p<.05$) percentages of B220+B7-1+ cells compared to wild-type ($n=3$).

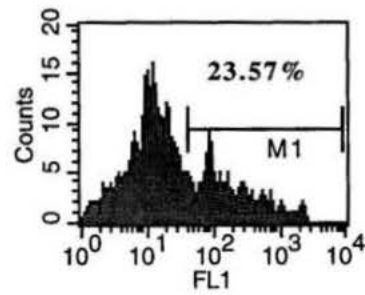
A.

NORMAL



B7-1+

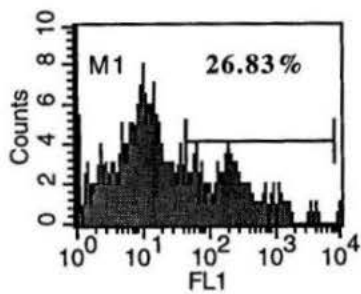
SCURFY



B7-1+

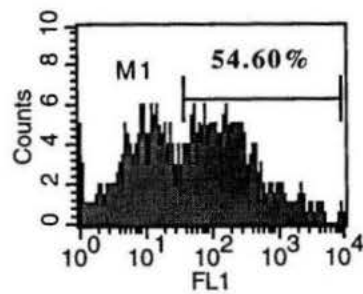
B.

NORMAL



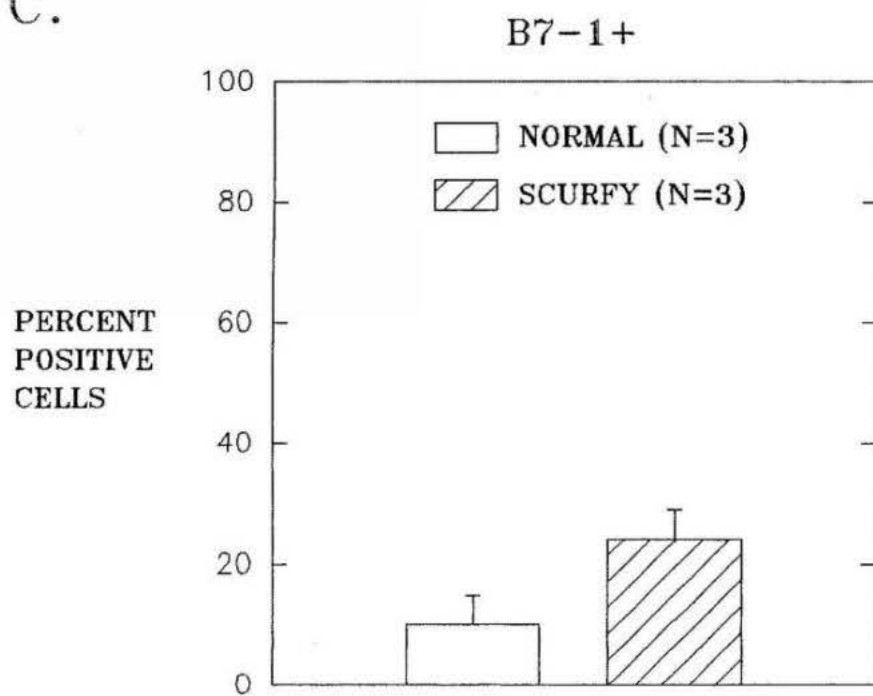
B7-1+/B220+

SCURFY



B7-1+/B220+

C.



D.

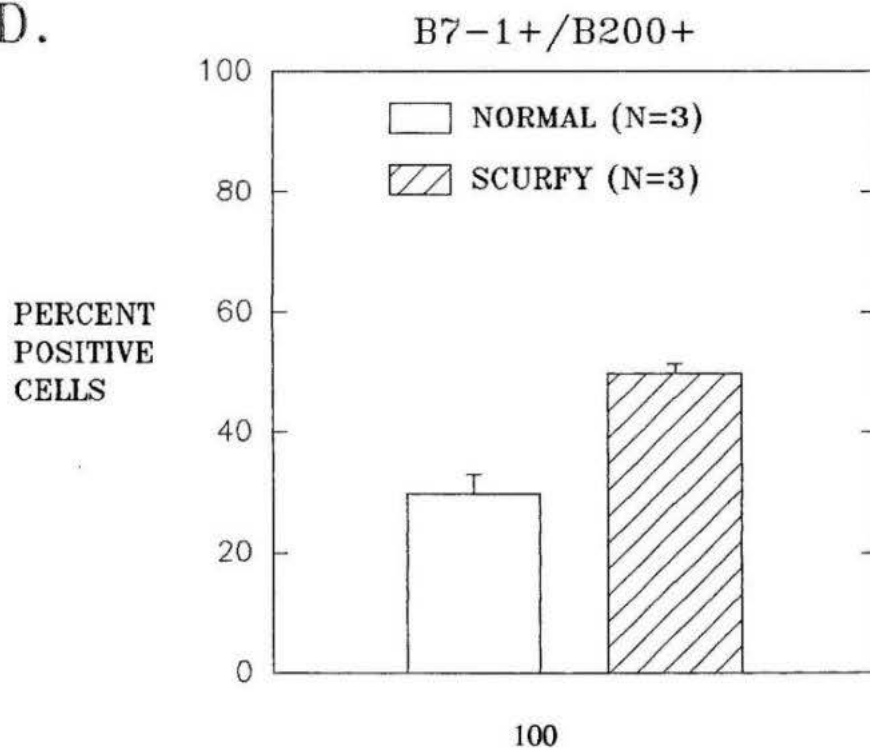
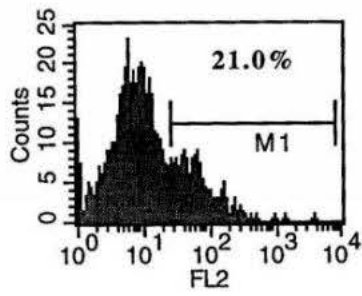


Figure 6. Flow cytometric analysis of B7-2 cell surface expression. Lymph node cells from day 19 wild-type and *scurfy* mice were stained for cell surface expression of B7-2 (CD86) as described in Methods and Materials. A representative sample of the data is shown in (A). Dead cells and debris were eliminated by gating on forward and side light scatter during data analysis. (B) The expression of B7-2 on whole lymph node cell populations was significantly increased ($p<.05$) in *scurfy* mice (n=3) compared to wild-type controls (n=3).

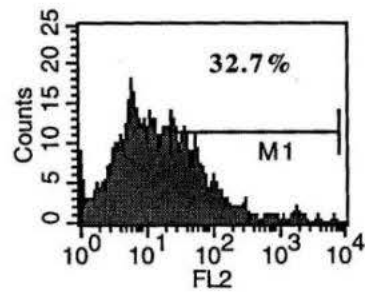
A.

NORMAL



B7-2+

SCURFY



B7-2+

B.

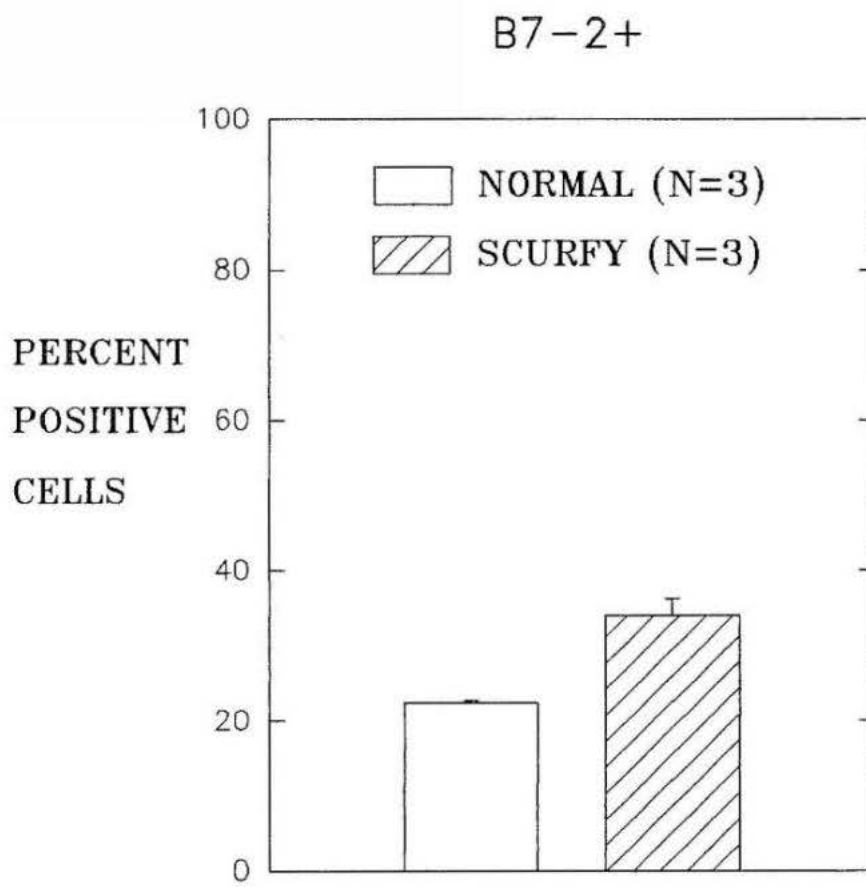
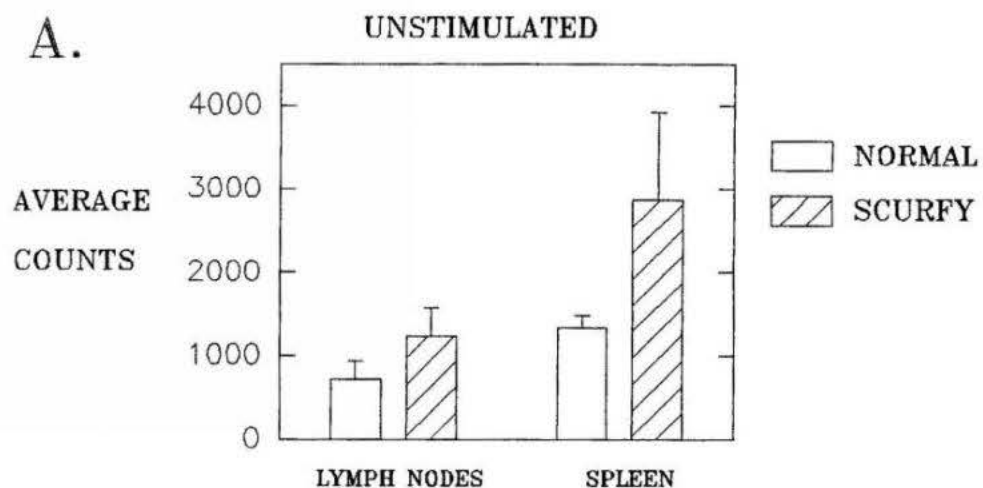
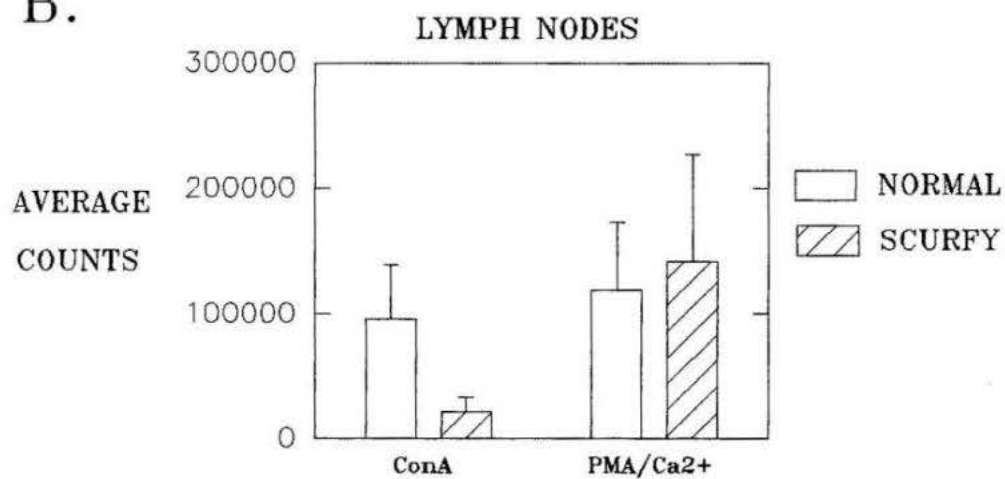


Figure 7. *In vitro* mitogen proliferation assays. Lymph node cells and splenocytes were cultured in media alone, with ConA (5ug/ml) or with PMA (15ng/ml) and calcium ionophore A23617 (250ng/ml) for 48 hours at 37° C, and pulsed for 8 hours with 1μCi of [³H]thymidine. (A) *Scurfy* (n=9) and wild-type (n=9) lymph node cells and splenocytes were cultured in medium alone, and their spontaneous incorporation of tritium measured. (B) shows the average incorporation of tritium of *scurfy* (n=4) and wild-type (n=4) lymph node cells in response to ConA and PMA/Ca²⁺. (C) shows the average incorporation of tritium of *scurfy* (n=5) and wild-type (n=5) splenocytes to ConA and PMA/Ca²⁺.

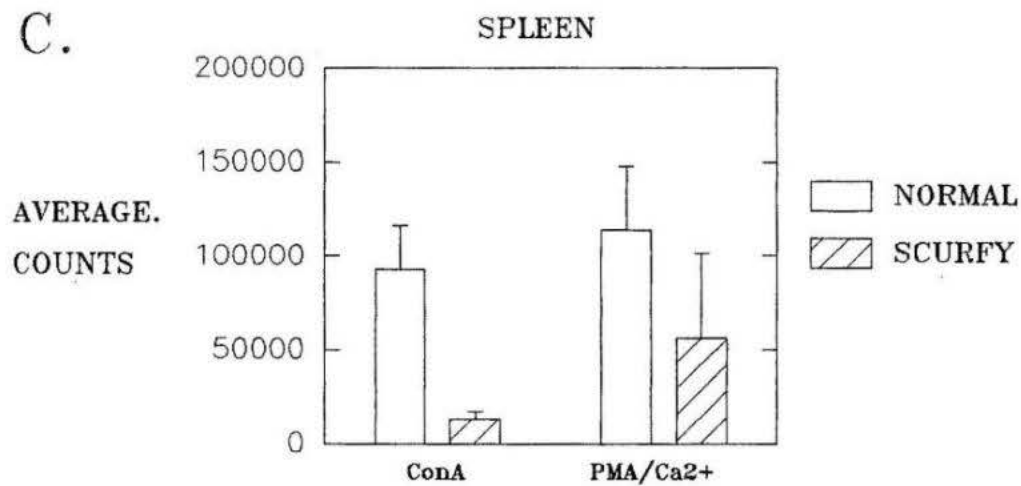
A.



B.



C.



Part 4

General Discussion and Summary

Discussion

Apoptosis

The massive lymphoproliferation observed in *scurfy* mice suggests a breakdown in peripheral homeostasis and immune tolerance. The first level of immune tolerance begins with selection in the thymus, resulting in self-tolerant, antigenically-reactive, mature T cells. Previous experiments investigating thymic selection in *scurfy* mice did not reveal any gross abnormalities (Blair *et al.*, unpublished data). Therefore we initiated experiments to examine pathways known to play a role in peripheral lymphocyte homeostasis and tolerance, namely the Fas/FasL pathway (Part 2) and the CD28/CTLA-4/B7 pathway (Part 3). Mice with defects in these pathways exhibit phenotypic similarities to *scurfy* mice, suggesting that the *scurfy* protein may function in one of these known lymphoid signalling pathways (Chu *et al.*, 1994; Takahashi *et al.*, 1994; Tivol *et al.*, 1995). We report here that both the Fas/FasL and CD28/CTLA-4/B7 pathways are activated in *scurfy* mice, and that *scurfy* lymphocytes can undergo apoptosis in response to signalling through the Fas receptor.

The main mechanism involved in peripheral lymphocyte homeostasis is deletion through apoptosis. This is achieved by the elimination of activated T cells by apoptosis at the conclusion of an immune response, and the elimination of self-reactive T cells in the periphery that have escaped thymic selection. Evidence suggests that there are two types of lymphocyte apoptosis involving distinct, nonoverlapping signalling pathways. The best

characterized apoptotic pathway is the Fas/FasL pathway. This pathway plays an obligatory role in the activation-induced cell death (AICD) of lymphocytes in response to chronic activation by self or foreign antigens encountered in the periphery (Gillette-Ferguson and Sidman, 1994). A defect in this pathway results in lymphoproliferation and autoimmune disease, characterized by the autosomal recessive mouse mutations *lpr* and *gld*, mutations in Fas and FasL, respectively (Chu *et al.*, 1994; Takahashi *et al.*, 1994). There is both *in vitro* and *in vivo* evidence that activation is a prerequisite for Fas-mediated apoptosis and the decision to proliferate or die in response to TcR ligation is intricately tied to the differentiation and activation states of lymphoid cells (Lenardo, 1991; Daniel *et al.*, 1994). Flow cytometric analysis of *scurfy* T lymphocytes revealed evidence of previous and recent activation, characterized by the cell surface phenotype - CD44^{high}, Mel-14^{low}, CD45RB^{low}, CD69+, CD25+. The source of lymphocyte activation in *scurfy* mice is unknown. It could result from incomplete thymic selection resulting in the release of potentially self-reactive T cells into the periphery, or it could result from inadequate termination of normal ongoing immune responses. In a normal state, these activated lymphocytes would be eliminated by Fas-mediated apoptosis. Their accumulation in *scurfy* mice suggests a potential defect in this pathway.

In addition to activation, Fas-mediated apoptosis requires the upregulation of cell surface Fas and the expression of FasL (Hanabuchi *et al.*, 1994). Northern Blot analysis detected increased levels of *Fas* mRNA in *scurfy* peripheral lymphoid organs, and RT-PCR analysis revealed constitutive expression of *FasL* mRNA in *scurfy* spleens. Constitutive

expression of *FasL* has been reported in mice with defects in the Fas-FasL pathway (Watanabe *et al.*, 1995; Chu *et al.*, 1995). Increased levels of Fas cell surface expression were also observed on *scurfy* CD4⁺ and CD8⁺ T cells and B220⁺ B cells by flow cytometric analysis. The upregulation of *Fas* and *FasL* mRNA, and Fas cell surface expression in *scurfy* lymphoid organs is consistent with their activated phenotype, and suggests that the lymphoid cells accumulating in *scurfy* lymph nodes are primed to undergo Fas-mediated apoptosis.

Although it appears that a large percentage of *scurfy* lymph node cells are primed for apoptosis, only 1.3% of freshly isolated *scurfy* lymph node cells were undergoing apoptosis *in vivo*. This value is not different from that observed for wild-type controls (1.4%). This supports our theory that *scurfy* lymph node cells have initiated the steps for Fas-mediated apoptosis, but are not completing the death program. In contrast, there was significantly increased percentages of freshly isolated *scurfy* thymocytes undergoing apoptosis compared to wild-type controls. However, this is most likely *scurfy* CD4⁺CD8⁺ double-positive thymocytes undergoing apoptosis in response to stress-induced production of glucocorticoids, which proceeds by a Fas-independent pathway (Memon *et al.*, 1995). This is supported by the dramatic decrease in CD4⁺CD8⁺ double-positive thymocytes observed in *scurfy* mice.

To determine if *scurfy* lymphoid cells are capable of Fas-mediated apoptosis, we injected a single large dose of purified anti-Fas mAb into wild-type and *scurfy* mice. Previous

reports have demonstrated that *in vivo* and *in vitro* treatment with anti-Fas mAb induces apoptosis in thymocytes, but not in the mature lymphoid populations of the lymph nodes and spleen (Ogasawara *et al.*, 1995; Trauth *et al.*, 1989). Our results with wild-type mice were in agreement with this data, with apoptosis detectable in anti-Fas-treated thymi, but not in anti-Fas-treated lymph nodes. In contrast, both *scurfy* thymi and lymph node cells were susceptible to Fas-mediated apoptosis. Therefore, if provided with the signal, *scurfy* lymphoid cells can undergo Fas-mediated apoptosis. Why they are not undergoing apoptosis *in vivo* under physiological conditions is not clear. However, there are a number of possible hypothesis: (1) *Scurfy* mice are receiving a negative signal that is blocking Fas-mediated apoptosis, and the amount of injected antibody is great enough to overwhelm this signal. (2) Even though *FasL* mRNA is detected in unstimulated *scurfy* spleens, there may not be sufficient levels of cell surface *FasL* on *scurfy* lymphoid cells. In fact, high glucocorticoid levels have been reported to inhibit the activation-induced expression of *FasL* (Yang *et al.*, 1995). In the absence of *FasL*, anti-Fas mAb induces apoptosis upon binding to the Fas receptor. *FasL* mAb is now commercially available, and *FasL* cell surface expression on *scurfy* lymphoid cells should be characterized. (3) It is also possible that *scurfy* have upregulated one or more lymphoid survival genes, such as *bcl-2* or *bcl-xl*, both of which have been implicated in blocking Fas-mediated apoptosis (Boise *et al.*, 1995).

In addition to Fas-mediated apoptosis, there is evidence for a second major apoptotic mechanism functioning in the periphery. This type of lymphoid death is referred to as

programmed cell death (PCD) and results from the lack of a costimulatory signal or from cytokine withdrawal or deprivation (Parijs *et al.*, 1996). This pathway is distinct from Fas-mediated AICD in that *lpr/lpr* mutants (Fas-deficient) still undergo this type of apoptosis. *In vitro* experiments in which naive murine lymph node cells were cultured in the absence of stimulation or exogenous cytokines, resulted in the apoptosis of a large percentage of lymphoid cells in both wild-type and *lpr/lpr* mice. In contrast, naive T cells stimulated through their TcR and CD28, showed enhanced survival in culture, which was linked to the expression of bcl-xl (Parijs *et al.*, 1996). Bcl-xl is a member of a family of proteins involved in promoting cell survival, and its dimerization with bax has been reported to prevent apoptosis (Boise *et al.*, 1993; Oltvai *et al.*, 1993). CD28 costimulation also enhances cytokine secretion, upregulating bcl-2 expression, which has also been implicated in promoting cell survival (Nunez *et al.*, 1990). To examine this pathway in *scurfy* mutants we cultured wild-type and *scurfy* lymph node cells and thymocytes for 16 hours in the absence of stimulation or exogenous cytokines. Increased percentages of both *scurfy* thymocytes and lymph node cells spontaneously underwent apoptosis in culture compared to wild-type. Increased spontaneous *in vitro* apoptosis has also been reported in *lpr/lpr* mutants (Houten and Budd, 1992). These results suggest that this second peripheral apoptotic pathway is intact in *scurfy* mice. *Scurfy* mice overexpress a number of cytokines and the increased kinetics of *scurfy* spontaneous apoptosis may reflect rapid cytokine deprivation upon *in vitro* culture (Blair *et al.*, 1994). Alternatively, the increased apoptosis observed in *scurfy* lymphoid cells could result from the removal of an inhibitory signal in the *scurfy* microenvironment. As previously

mentioned, *scurfy* lymphoid cells are phenotypically primed for Fas-mediated apoptosis, but we found no evidence that they are dying *in vivo*. Perhaps removing them from the *scurfy* microenvironment eliminates an inhibitory signal and they proceed to finish the apoptotic pathway. Another possibility is that *in vivo*, a number of cell survival proteins are upregulated, perhaps due to chronic stimulation through CD28, protecting these cells from apoptosis. When cultured *in vitro*, the expression of these cell survival proteins is rapidly lost, and the cells become susceptible to apoptosis. The rapid loss of bcl-2 expression upon *in vitro* culture has been documented for murine lymphoid cells (Nunez *et al.*, 1994). Therefore, our data suggest that the accumulating activated lymphoid cells in *scurfy* mice are primed for apoptosis, but for unknown reasons are not undergoing apoptosis *in vivo*. It is not because of an intrinsic defect in the Fas-FasL pathway that *scurfy* lymphoid cells are not undergoing apoptosis. Signalling through the Fas receptor with anti-Fas mAb results in rapid apoptosis in both *scurfy* thymocytes and lymph node cells. We hypothesize that apoptosis is blocked either by an inhibitory signal in the *scurfy* microenvironment, or by the upregulation of cell survival proteins such as bcl-2 and bcl-xl.

CD28/CTLA-4/B7

In addition to the Fas/FasL pathway, the CD28/CTLA-4/B7 pathway has been found to be involved in maintaining peripheral T cell homeostasis. The lymphoproliferative phenotype of mice with a null mutation in CTLA-4 revealed that CTLA-4 is not a redundant costimulatory receptor, but plays an obligatory role in lymphocyte homeostasis.

In the absence of CTLA-4, the immune system is massively activated, resulting in early lethality due to multiorgan lymphocyte infiltration (Tivol *et al.*, 1995). The phenotype described for CTLA-4^{-/-} mice is almost identical to that of *scurfy*. Therefore, it is possible that *scurfy* functions upstream or downstream of CTLA-4 in the CD28/CTLA-4/B7 pathway. Our data demonstrates that *scurfy* lymphoid cells have increased intracellular stores of CTLA-4 protein, characteristic of activated T cells. In addition, *scurfy* T cells have increased CTLA-4 cell surface expression, limited to the CD4⁺ T cell subset. This suggests that if *scurfy* functions in this pathway, it is downstream of CTLA-4 expression. There have been reports of a third CD28/CTLA-4 ligand, distinct from the two documented B7 ligands (Boussiois *et al.*, 1993). The nearly identical phenotypes of *scurfy* and the CTLA-4^{-/-} mutants implies that perhaps *scurfy* is this unknown CTLA-4-specific ligand.

Although the downstream signalling pathways of CTLA-4 are largely unresolved, they are thought to involve activation of the Ras signalling pathway. The Ras pathway is constitutively activated in CTLA-4^{-/-} T cells, and downstream effectors such as the mitogen-activated protein kinase (MAPK) are also constitutively activated in the absence of CTLA-4 (Marengere *et al.*, 1996). Although Ras signalling is generally implicated in promoting cell survival and proliferation, it has been found to be a necessary, although not sufficient, step in Fas-mediated apoptosis (Gulbins *et al.*, 1995). This implies that there are two distinct pathways after Ras activation: one leading to cellular activation and proliferation, and the other leading to apoptosis. Perhaps *scurfy* is responsible for

determining which pathway prevails after TcR ligation and Ras activation: (1) an inhibitory pathway characterized by negative signals through CTLA-4 and apoptosis through Fas; or (2), a proliferative pathway involving positive signals through CD28 and the MAPKs, resulting in activation and proliferation.

Summary

We report here that *scurfy* T cells exhibit phenotypic characteristics of previously and recently activated lymphocytes. In addition, *scurfy* peripheral lymphoid cells upregulate both *Fas* mRNA and cell surface protein, and constitutively express *FasL* mRNA. When signalled through the Fas receptor, *scurfy* lymphoid cells can initiate Fas-mediated apoptosis, although why they do not *in vivo* is not clear. In addition, *scurfy* T cells have upregulated both surface and intracellular CTLA-4, and have increased percentages of lymph node cells expressing the B7-1 and B7-2 ligands. Therefore, in addition to the activation of the Fas/FasL pathway, *scurfy* have also activated the CD28/CTLA-4/B7 pathway. This data in connection with the similar lymphoproliferative phenotypes of *scurfy* and CTLA-4^{-/-} mice suggests that *scurfy* functions in the CD28/CTLA-4/B7 pathway or an overlapping pathway. If the *scurfy* defect is in the CD28/CTLA-4/B7 pathway, our data implies that the defect must lie downstream of CTLA-4 expression.

In conclusion, we favor a scenario, parts of which are supported by the data in this report, in which *scurfy* T cells are receiving chronic signalling through CD28, resulting in the upregulation of cell survival proteins such as Bcl-2 and Bcl-xl. Because of a defect in

CTLA-4 or an overlapping pathway, *scurfy* T cells do not receive a down-regulatory signal. *Scurfy* lymphoid cells are therefore constantly activated, resulting in cytokine production, B cell activation and antibody production. T cell activation upregulates Fas and FasL, but Fas-mediated apoptosis is blocked by the upregulation of Bcl-2 and Bcl-xl. The accumulation of activated lymphoid cells is ultimately responsible for the cytokine toxicity and early lethality observed in *scurfy* mice. Future experiments will examine the expression of FasL, Bcl-2, and Bcl-xl on *scurfy* T lymphocytes in an attempt to discover why *scurfy* lymphocytes are not undergoing Fas-mediated apoptosis under physiological conditions.

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